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Toxigenic Clostridia

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INTRODUCTION

Clostridia are obligately anaerobic, sporeforming bacilli that usually stain gram positive, at least in the early stages of growth (54). Anaerobic bacteria can be defined as organisms that "(i) generate energy and synthesize their substance without recourse to molecular oxygen; and (ii) demonstrate a singular degree of adverse oxygen sensitivity which renders them unable to grow under an atmosphere of air" (207). Although some species of *Clostridium* do grow in the presence of air, they can be distinguished from *Bacillus* species by their lack of catalase (298). They lack both catalase and peroxidase, which protect biological systems against the deleterious effects of oxygen, and they lack cytochrome oxidase, which is involved in aerobic metabolism (71, 207). Superoxide dismutase is present in some clostridia (134). Members of the genus are found in soil, sewage, marine sediments, and the intestinal tracts of humans and other animals and in decaying animal and plant products (54). Because of the ubiquity of these organisms, they find their way into wounds, foods, and feeds and often are responsible for serious illness, usually (if not always) mediated by their toxins.

The genus *Clostridium* consists of organisms with G+C content of 22 to 55 mol%, indicating a great diversity of genomes. Cato et al. (54) suggest that it might someday be divided into two genera; one with species having 22 to 34 mol% G+C, and the other containing the species with greater than >40 mol%. The species covered in this review have a G+C content range of 24 to 29 mol%. Of the 83 species of *Clostridium* listed in *Bergey's Manual of Systematic Bacteriology* (54), about 20 are pathogenic or otherwise encountered in clinical or environmental specimens pertaining to illness or infections in humans or other animals (298). Fourteen species that are clearly or potentially pathogenic in and of themselves produce biologically active proteins that are responsible for their pathogenicity. These organisms are listed in Table 1, along with their major toxins and their activities and the diseases they cause. This review will discuss the present state of our knowledge of these organisms and their toxins and, when known, their mechanism of action, the source of the genetic information that codes for them, and, when applicable, the means of toxin detection that provide laboratorians with methods for investigation and confirmation of the various diseases.

The term toxin with respect to the genus *Clostridium* refers to biologically active proteins that are antigenic in nature, and thus their activities can be specifically neutralized with appropriate antisera. They have molecular masses ranging from 22 to 600 kilodaltons (kDa) (Table 1). They are

TABLE 1. Toxigenic clostridia and their toxins

Species	Toxins	Size of molecule (kDa)	Activity/disease
C. botulinum	Neurotoxin	150	Botulism
	C_2 (binary)		Permease
	Component I	50	ADP-ribosylation
	Component II	105	Binding
	C_3	25	ADP-ribosylation
C. argentinense (*C. botulinum* type G)	Neurotoxin	ND[a,b]	Botulism (experimental)
C. tetani	Neurotoxin	150	Tetanus
	Tetanolysin	48	Oxygen-labile hemolysin
C. perfringens	Major		
	Alpha	43	Phospholipase C/myonecrosis
	Beta	40	Lethal, necrotic/enterotoxemia
	Epsilon	34	Lethal, permease/enterotoxemia
	Iota (binary)		Enterotoxemia
	Component a	48	ADP-ribosylation
	Component b	72	Binding
	Other		
	Enterotoxin	35	Foodborne diarrhea
	Delta	42	Hemolysin
	Theta	51	Oxygen-labile hemolysin
	Kappa	80	Collagenase
	Lambda	ND	Protease
	Mu	ND	Hyaluronidase
	Nu	ND	DNase
	Neuraminidase	43, 64, 105, 310	*N*-Acetylneuraminic acid glycohydrolase
C. difficile	Toxin A	400–500	Enterotoxin/AAPMC[c]
	Toxin B	360–470	Cytotoxin/AAPMC
	CDT	43	ADP-ribosylation
C. sordelii (*C. bifermentans*)	Alpha	43	Phospholipase C
	Beta		Lethal
	HT	525	Equivalent to *C. difficile* toxin A
	LT	250	Equivalent to *C. difficile* toxin B
	Hemolysin	43	Oxygen-labile hemolysin
C. novyi/*C. haemolyticum*	Alpha	260–280	Lethal
	Beta	32	Phospholipase C
	Gamma	30	Phospholipase C
	Delta	ND	Oxygen-labile hemolysin
	Epsilon	ND	Lipase
C. chauvoei/*C. septicum*	Alpha	27	Lethal, necrotizing
	Beta	45	DNase
	Gamma	ND	Hyaluronidase
	Delta	ND	Oxygen-labile hemolysin
C. histolyticum	Alpha	ND	Necrotizing
	Beta		Collagenases
	Class I	68, 115, 79, 130	
	Class II	100, 110, 125	
	Gamma	50	Proteinase, thiol-activated
	Delta	>10, <50	Proteinase
	Epsilon	ND	Oxygen-labile hemolysin
C. spiroforme	Iota (binary)		Diarrhea in rabbits
	Component a	43–47	ADP-ribosylation
	Component b	ND	Binding
C. butyricum	Neurotoxin	145	Botulism, type E
C. baratii	Neurotoxin	ND	Botulism, type F

[a] ND, Not determined.
[b] For neurotoxin of *C. argentinense*, a 16S complex of 500 kDa has been purified, but the size of its toxic subcomponent has not been determined (227).
[c] AAPMC, Antibiotic-associated pseudomembranous colitis.

TABLE 2. Mouse lethal doses of selected clostridial toxins

Species	Toxin	Mouse LD_{50} (ng)[a]	Reference
C. botulinum type A	Neurotoxin	0.00625 (i.p.)	274
C. tetani	Neurotoxin	0.015 (i.p.)	133
C. perfringens types B and D	Epsilon	0.32	110
C. perfringens types B and C	Beta	8	276
C. difficile	Toxin A	26 (i.p.)	16
C. botulinum type C	C_2	45 (i.p.)	238
C. perfringens type A	Alpha	50	327
C. perfringens types B and C	Delta	60	7
C. perfringens type A	Theta	167	352
C. perfringens type A	Enterotoxin	1,400	304
C. difficile	Toxin B	1,500	16
C. perfringens type E	Iota	1,560	308
C. bifermentans	Lecithinase	2,500	327
C. perfringens type A	Kappa	30,000	152

[a] Dose was determined by i.v. injection unless indicated otherwise; i.p., intraperitoneal. Published values expressed in terms of *N* were converted to protein by multiplying by 6.25; those in mean lethal doses were converted to 50% lethal doses (LD_{50}) by multiplying by 0.5; and those expressed per kilogram of body weight were recalculated for 25-g mice.

detected by their effects in whole animals, tissues, or cultured cells or by reactions in biochemical tests. The dominant feature of many clostridial toxins is their lethality for animals. A comparison of the lethality of some of the toxins is provided in Table 2, showing a range in lethal dose for mice of 0.006 to 30,000 ng.

Some of the toxins such as the neurotoxins are so obvious in their pathological role that they tend to eclipse most other features of the organisms that produce them. These toxins have names indicating the illnesses they cause, and the toxin dictates the nomenclature for the organisms. Thus, diverse organisms sometimes bear a common name. Detecting the toxin in the patient or stricken animal has sometimes rendered the isolation of the organism unnecessary. Secondary toxins produced by these organisms have been neglected to some extent.

Toxin differences that are responsible for significantly different pathology have in some cases dictated separate species names for genetically similar organisms. Toxins with subtle or less obvious activities have been subordinate to the organisms that produce them and generally have provided means of differentiating groups or toxin types. Most of these distinctions have been possible because toxins could be demonstrated by differential neutralization, before a precise activity was understood. For each species, these toxic factors were generally designated by Greek letters, assigned in the order of their discovery. Thus, no relation usually exists between toxins of different species designated by the same Greek letter. Notable exceptions are the iota-toxin of *Clostridium perfringens* and *C. spiroforme* and the toxins produced in common by the closely related organisms *C. novyi* and *C. haemolyticum* and *C. chauvoei* and *C. septicum*. It is perhaps unfortunate that the term toxin continues to be used for antigenic factors that are not, in fact, toxic. Also, the use of different Greek letters to indicate the same or very similar biologically active proteins produced by different organisms is confusing. These problems will not be solved in the writing of this review, but it seems that these problems need to be dealt with by a committee of experts on the organisms in the genus *Clostridium*.

The subject of this review is broad and the literature is so vast that only a selected sample of the contributions of the many researchers can be cited. Some of the toxins have been well characterized by work begun even before the turn of the century and continuing up to the present. Other toxins have been discovered only recently but have attracted intensive interest, and the work has quickly yielded much progress. Others have been recognized for many years, but their nature remains obscure because of difficulties in isolation and purification or because they have been neglected for other reasons. Therefore, the treatment of the various organisms and their toxins will not be uniform. Because of the recent review in this journal entitled "*Clostridium difficile*, Its Disease and Toxins" (178), the coverage of this important subject will be abbreviated.

REFERENCES, TEXTS, AND REVIEWS

The chapter on *Clostridium* in *Bergey's Manual* (54) serves as the starting point for reference to the clostridial species and their characteristics. This source is supplemented and amplified by the reference work of Holdeman et al. (139, 206) and the texts of Smith and Williams (298) and Willis (345). The latter is dated, but is an excellent source of the earlier information on the bacteriology, toxins, and diseases of clostridia. An extensive coverage of the bacteriology and pathology of gas gangrene is presented by MacLennan (184). The monograph "Clostridia in Gastrointestinal Disease," edited by Borriello (39), provides reviews and literature sources on most of the clostridial diseases of current concern.

On the subject of clostridial neurotoxins, reviews are available by Habermann and Dreyer (112) and Simpson (292) on the molecular properties and pharmacological action of tetanus and botulinal toxins and in a monograph containing contributions on many aspects of these toxins edited by Simpson (293). An older text of historic interest concerning tetanus was written by Adams et al. (1), and a more recent monograph on various aspects of tetanus ranging from microbiological and biochemical to clinical and public health interests was edited by Veronesi (337). In addition, Bizzini (31) compiled a review on the biochemistry of the toxin and mode of action of tetanus toxin, and Bleck (33) has provided a review on the pharmacology of tetanus. Smith and Sugiyama have recently updated the most comprehensive text on botulism (297), and a monograph edited by Eklund and Dowell (83) was published on avian botulism with major emphasis on *C. botulinum* types C and D and the toxins they produce. Two other reviews on botulinal toxins, by Sugiyama (315) and Sakaguchi (274), are available. Several reviews are available on *C. perfringens*: on the enterotoxin by McDonel (194), Granum (104), and McClane et al. (187); on the various toxins and virulence factors by Smith (296) and McDonel (193); and on the diseases of veterinary interest by Niilo (222). Finally, a review of *C. difficile* was written by Lyerly et al. (178) that appeared in the initial issue of this journal.

CLOSTRIDIUM BOTULINUM

Botulism

Foodborne botulism. Botulism is generally thought of as a foodborne intoxication (297). The earliest scientific considerations of the disease were published by Kerner in about 1822 (cited by Smith and Sugiyama [297]). The bacterial etiology and toxicopathic nature of the disease were elucidated in 1895 by van Ermengem in his investigation of an outbreak of botulism in Ellezelles, Belgium, that involved 50 cases (171), 3 of them fatal and at least 10 of them very serious but not fatal (333). The clinical features and postmortem findings were reported in detail. The outbreak was caused by a salt-cured uncooked ham. Portions of macerated ham fed to mice, guinea pigs, and monkeys caused paralytic signs of the illness observed in the patients and subsequently resulted in death of the animals; feedings to rats and cats had little effect, but subcutaneous injection of macerated ham caused symptoms and death. Dogs and chickens were unaffected by feedings or injections. Filtered extracts of the ham had the same effects as the macerated ham. Van Ermengem consistently found an anaerobic sporulating bacillus in cultures of the ham as well as in a culture of spleen from one of the decreased victims. Cultures and culture filtrates of the organism had the same effects on susceptible animals as did the ham macerates. The organism was named *Bacillus botulinus*.

Wound botulism. In 1943, a fatal, progressive, flaccid, paralytic illness was observed in a 15-year-old girl who had severely broken both legs in a fall from a building; the case was diagnosed as botulism (69). *C. botulinum* type A was recovered from a mixture of organisms cultured from the infected wound. The evidence suggested that botulism could result from the elaboration of the toxin by the organism growing in a wound, analogous to the mode of intoxication in tetanus. A review of the initial case and eight subsequent confirmed cases was published by Merson and Dowell (197). More recently, MacDonald et al. (179) described two cases of confirmed botulism and four cases of botulismlike illness associated with chronic drug abuse. Five patients used drugs parenterally, and the sixth patient had maxillary sinusitis, possibly secondary to heavy cocaine abuse, which might have served as a site of infection by toxigenic organisms. Through 1987, approximately 40 cases of wound botulism have been documented in the United States (Centers for Disease Control, unpublished data).

Infant botulism. A third form of botulism, designated infant botulism, was recognized in 1976 (201, 249). Botulism paralysis results from absorption of toxin produced by the toxigenic organisms colonizing the intestinal tract of certain infants under 1 year of age. Since its discovery, this form of the disease has become the most common form confirmed in the United States, with as many as 99 cases reported in 1 year (120). The belief is that affected infants are colonized because they have not yet established a stable normal gut flora that would inhibit the germination of ingested spores and outgrowth of the organisms. One source of the spores is honey (11), but many patients have no known exposure to honey. By the age of 1 year, the susceptibility to colonization appears to have passed. The similarity in age group of infant botulism patients and sudden infant death syndrome prompted investigations on whether some cases of sudden infant death syndrome were due to undiagnosed botulism. Arnon et al. (12) found that intestinal specimens from 10 (4.7%) of 212 cases of unexplained death in infants yielded *C. botulinum* in culture; specimens from two contained botulinal toxin demonstrable in mouse tests. A study in Switzerland reported evidence of botulism in nine cases of sudden infant death syndrome (299). These results are difficult to accept without question because seven of the cases involved toxin types C, F, and G and because no hospitalized cases of infant botulism had been recognized up to the time of that study. Type G botulism has not been documented in humans or animals elsewhere, while type F is extremely rare and human type C botulism is almost unknown (see below). The first case of infant botulism in Switzerland was subsequently confirmed in Switzerland in 1987, and it was caused by toxin type A (97). Other studies on sudden infant death syndrome, including 88 cases in the United States (Centers for Disease Control, unpublished data), 41 cases in Germany (130), and 33 cases in Canada (109), failed to find evidence that any were due to botulism.

Intestinal colonization of adults. A form of botulism similar to infant botulism but occurring in adults (or older children) has been proposed from time to time, and evidence in a few rare cases indicates that it does occur (191). Irrefutable evidence of colonization was documented in the case of a 37-year-old patient whose stools yielded the toxigenic organism as long as 119 days after diagnosis of botulism (59, 191). No toxic food was incriminated, but a coconut product consumed by the patient was found to contain spores of *C. botulinum* of the same toxin type as found in her stools. The patient had previously had gastrointestinal surgery, which could have modified conditions in the gut so that spore germination and outgrowth of the microorganisms were favored.

Botulism paralysis. Regardless of the manner in which botulinal toxin gains access to the body system, ingestion of preformed toxin or elaboration of toxin by the organism in a wound or the gastrointestinal tract, the mechanism of paralysis is identical and the manifestations of the disease are generally the same. Of the latter, differences certainly exist in gastrointestinal features among foodborne, infant, and wound forms of the disease. Nausea and vomiting are frequent in foodborne (56) and nonexistent in infant (175) botulism, while constipation is a prominent feature in infant botulism. Toxin in the gut finds its way into the circulation via the lymphatic system. Toxin is demonstrable in the serum of about 35% of foodborne (78) and wound (121) botulism cases. It can also be detected in sera of infants with botulism, more frequently with type A (36%) than with type B (2%) (122). The toxin binds to receptors on the peripheral nerve endings, blocking synapses to muscle fibers (292, 315). After the initial binding step, a portion of the bound toxin molecule is internalized and by an unknown process prevents the release of acetylcholine from the nerve ending. Thus, muscle response to a nerve impulse is blocked because no neurotransmitter is released to excite the muscle. Recovery of nerve function requires regeneration of new nerve endings.

Diagnosis and confirmation of botulism. Botulism is first diagnosed on the basis of the patient's signs and symptoms, and foodborne incidents sometimes can be corroborated by food history and epidemiological evidence. Botulism can be confirmed by detecting toxin in patient serum or stool or in a food sample. *C. botulinum* isolated from a wound specimen is good evidence for wound botulism, and isolation of the organism from fecal samples is generally satisfactory for confirming foodborne or infant botulism. Although enzyme-linked immunosorbent assays for detecting botulinal neurotoxins have been devised (72, 224, 225), the mouse bioassay

is still generally used for diagnostic investigation of botulism. Detailed laboratory procedures for toxin detection and recovery of toxigenic organisms are given on the package insert distributed with the Centers for Disease Control diagnostic reagents and have been published elsewhere (120, 155).

The Organisms

Toxin types. In 1910, Leuchs reported that toxin from an organism responsible for an outbreak of botulism in Germany due to beans (163) could be distinguished from the toxin of the van Ermengem strain of *Bacillus botulism* by differential neutralization with specific antisera (171). In 1919, type A and type B designations of toxin types were established, using strains isolated in investigations of botulism outbreaks in the United States (48). Although the van Ermengem and Landman strains were no longer available, it is believed that the toxin of the former would correspond to type B and that of the latter would correspond to type A (199). Organisms causing botulism in chickens in the United States (22) and cattle in Australia (286) were found to be of a new toxin type, designated C. It was noted, however, that while the antitoxin prepared with the organism isolated from chickens (Bengtson strain) neutralized the lethality for mice of both strains, the antitoxin against the Australian isolate (Seddon strain) neutralized only the homologous toxin. Gunnison and Meyer (108) suggested the subtypes C-alpha and C-beta to designate toxin from those respective strains. The genus name *Clostridium* was adopted in preference to *Bacillus* in accordance with the recommendation of the Society of American Bacteriologists that the former would include anaerobic sporeformers and the latter would include only aerobic organisms (23).

An outbreak of botulism in cattle in South Africa was due to an organism similar to type C, but its toxin was immunologically distinct. This was designated toxin type D by Meyer and Gunnison (198, 200). *C. botulinum* type E was discovered as the causative agent in two outbreaks in 1934, one in New York State due to canned herring from Germany (128) and in Dnieptopetrovsk, the Ukraine, also due to fish (297). Gunnison et al. (106) established that the Ukranian isolate was a new toxin type which they designated as type E, and Hazen (129) confirmed that the herring isolate toxin was neutralized by the same antitoxin. In 1960, yet another type (F) was discovered as the cause of botulism due to liver paste in Denmark (203). Finally, an organism was isolated from soil in Argentina that produced a botulinal neurotoxin not neutralizable by antitoxins A through F (100). This organism was designated *C. botulinum* type G.

Distribution and habitat. *C. botulinum* is commonly found in soil samples and aquatic sediments. Hauschild (125) has provided a comprehensive summary of studies on soil samples and aquatic sediments from North and Latin America, Europe, Asia, South Africa, Iceland, and New Zealand. He has attempted to provide an estimate of density of the spores (most probable number per kilogram of soil or sediment) for each area surveyed and lists the percentage of isolates of each toxin type. The spore densities in soils range from 1 to 6 (Great Britain) to 2,500 (Netherlands potato fields), and those in sediments range from 1 (Faroe Islands, Gulf of Maine) to 920 (Lake Michigan). Types A and B are predominant in the soils of the continental United States, but type E is the most common in soils of Alaska, northern Europe, and Japan and in aquatic sediments for which surveys have been made. Smith and Sugiyama (297) also cover the subject well in their second chapter on the natural occurrence of *C. botulinum*. It has been assumed that type B isolates from European soils are generally nonproteolytic (group II), since those organisms are often implicated in European outbreaks, but this is often not verified by cultural studies on soil and food isolates. Meyer and Gunnison (199) found that most of the type B isolates from Europe available for study were proteolytic. They suggested that, since the nonproteolytic organism is more difficult to recover from cultures of specimens, few of these organisms are actually isolated. Smith (295) performed an extensive survey of *C. botulinum* in the soils of the continental United States. In 260 soil samples, he found 26 that contained type A strains; 22, type B; 3, type C; 5, type D; and 6, type E. Type F strains have been isolated from marine sediments from the Pacific Coast (84) and from crabs from the Chesapeake Bay (344). Type G organisms have been isolated from two soil samples in Argentina (100; A. S. Ciccarelli, A. M. Giuletti de Rigo, and D. F. Gimenez, Primer Congreso y IV Jornadas de Microbiologia 1976, abstr. no. 21). Sonnabend et al. (300) reported isolation of type G strains from five soil samples in Switzerland.

Physiological characteristics of organisms producing botulinal neurotoxins. It was discovered early that the organisms capable of producing botulinal toxins were diverse. Leuchs (171) noted that the van Ermengem strain had a lower optimum growth temperature than the Landman (or Darmstadt) strain. The former was nonproteolytic with respect to milk proteins by van Emmengen's original description (333), while the American strains studied by Burke when she established the toxin types A and B (48) were strongly proteolytic (23). Bengtson (23) found that the type C strains isolated from fly larvae associated with avian botulism and isolates from a chicken and a horse with botulism were nonproteolytic with respect to meat, milk, serum, and egg proteins. A European strain that produced type B toxin was also nonproteolytic, in contrast to the American type B strains. Differences in fermentative abilities of the groups of organisms were noted. The extent of growth of all nonproteolytic strains was correlated with the presence of a fermentable carbohydrate, while the proteolytic strains grew luxuriantly regardless of the presence or absence of a fermentable carbohydrate. The European type B strain fermented adonitol and inositol, and the type C strains fermented galactose and inositol, but none of these carbohydrates were fermented by the proteolytic strains. Type D toxin-producing strains were found to correspond physiologically to the strains that produce type C toxin (200). Organisms producing type E toxin were found to conform with the nonproteolytic type B toxin-producing organism isolated in Europe and studied by Bengtson (23, 128). Strains capable of producing type F toxin (mostly unrelated to botulism incidents) are rather evenly divided between proteolytic and nonproteolytic strains, resembling type A and E strains, respectively (R. T. Howell, Dr.P.H. dissertation, University of North Carolina, Chapel Hill, 1969). All of the organisms capable of producing botulinal neurotoxins of types A through F possess in common the ability to produce lipase, which can be detected as an iridescent film surrounding the bacterial growth on egg yolk agar medium (190). Overall, this characteristic has served well as a marker for isolating the various organisms known as *C. botulinum*. However, with the discovery of the most recent serological variant of botulinal toxin, type G, the system became more complex since the organism designated *C. botulinum* type G was not only lipase negative but also asaccharolytic (100). The name

TABLE 3. Characteristics of *Clostridium* spp. that produce botulinal neurotoxin

Group	Type(s) of toxin	Production of:		Carbohydrate fermentation	Gelatin hydrolysis	Casein digestion	Metabolic products[a]	
		Lecithinase	Lipase				Volatile	Nonvolatile
I *C. botulinum*	A, B, F	−	+	+	+	+	A, iB, B, iV	PP
II *C. botulinum*	B, E, F	−	+	+	+	−	A, B	
III *C. botulinum*	C, D	+	+	+	+	±	A, P, B	
IV *C. argentinense*	G	−	−	−	+	+	A, iB, B, iV	PA
Miscellaneous								
C. baratii	F	+	−	+	−	−	A, B	L
C. butyricum	E	−	−	+	−	−	A, B	

[a] As determined by gas-liquid chromatography. A, Acetic; P, propionic; iB, isobutyric; B, butyric; iV, isovaleric; PP, phenylpropionic (hydrocinnamic); PA, phenylacetic; L, lactic acid.

C. argentinense has been proposed for these organisms and their genetically related nontoxigenic counterparts (313).

Bengtson (23) has proposed making the distinction between proteolytic and nonproteolytic organisms (based on their ability to digest egg proteins) by designating two different species names, *C. parabotulinum* and *C. botulinum*, respectively. Later, a nomenclature subcommittee of the International Association of Microbiologists rejected the use of the species epithet *parabotulinum* and required that all organisms causing botulism be designated *C. botulinum* followed by a letter to indicate the toxin type (259). Distinction of variant strains based on proteolytic activity that produced type A and B toxins was to be made by appending var. *ovolyticus* or var. *nonovolyticus* to the name (e.g., *C. botulinum* B var. *nonovolyticus*). With the finding of additional toxin types, especially type G, an organism having such grossly different characteristics (100), and the discovery of neurotoxigenic organisms genetically identifiable as other established *Clostridium* species (312), the recommended nomenclature has become rather unwieldy.

The scheme for nomenclature in current use establishes four groups of *C. botulinum*, each designated by a Roman numeral (54, 297, 298). Group I includes the proteolytic strains of toxin types A, B, and F. Thus far, all reported strains that produce type A toxin belong in this group. Group II contains all toxin type E strains and the nonproteolytic strains of toxin types B and F. Group III contains strains commonly causing avian and nonhuman mammalian botulism that produce type C or D toxin. Group IV contains the organisms that produce type G toxin (*C. argentinense*). The non-neurotoxigenic *Clostridium* species, *C. sporogenes*, *C. novyi*, and *C. subterminale*, are phenotypically related to groups I, III, and IV, respectively. The differential characteristics for the four groups of organisms are shown in Table 3. Rare strains of other clostridia (*C. baratii* and *C. butyricum*) that produce botulinal neurotoxin have been found and are listed in a fifth group.

One problem with defining the species, *C. botulinum*, on the basis of toxigenicity is the instability of this characteristic for many strains (107). May a nontoxigenic isolate derived from a toxigenic strain retain the species name of its parent strain? Can one properly identify an organism that lost its toxigenicity just before isolation? Another problem has become apparent with the isolation of organisms readily identifiable by physiological characteristics as *C. baratii* (115) and *C. butyricum* (192), which produce botulinal toxin and have been implicated as causative agents of botulism. At this stage of our understanding of the diversity of organisms capable of producing botulinal neurotoxins, it may be more scientifically consistent and less confusing to allow distinct species names for organisms of each physiological group, whether the strain is toxigenic or not.

Genetic studies. The genetic relatedness of toxigenic strains of *C. botulinum* groups I, II, and III and their nontoxigenic counterparts and *C. argentinense* (group IV) has been the subject of several studies based on DNA homology (169, 170, 215, 217, 313, 349). In general, all strains, regardless of toxin type within group I or II, are closely related, while strains belonging to different physiological groups have low relatedness even though they may produce the same type of toxin. Phenotypically similar nontoxigenic organisms may show high, intermediate, or low genetic relatedness to their toxigenic counterparts. Heterogenicity is apparent among strains identifiable as *C. sporogenes*. Some are closely related to group I *C. botulinum*, while others are not (170, 217, 349). The type strain apparently is among the latter group (313). Some genetic diversity exists among strains in group III (169, 215). At least two genetic groups of toxigenic organisms are intermediately related to each other, as well as to *C. novyi* types A and B and *C. haemolyticum* (215).

The relationship between group IV organisms, *C. subterminale*, and *C. hastiforme* was studied by Suen et al. (313). All available strains of toxigenic organisms identified as *C. botulinum* type G were very closely related (>90%) to each other and were related also at the species level (>75%) to three nontoxigenic strains, two of them previously identified as *C. subterminale* and one previously identified as *C. hastiforme*. The type strains of the latter two species, however, were not related to the toxigenic organisms. The name *C. argentinense* was proposed for the organisms that produce type G botulinal toxin as well as for their nontoxigenic counterparts. A common identifying characteristic of the toxigenic and nontoxigenic strains of *C. argentinense* is their ability to produce an indole derivative. This nomenclature proposal was made as an attempt to begin to systematize the taxonomy of clostridia capable of producing botulinal neurotoxins.

All *C. argentinense* strains and strains of similar clostridia were analyzed by multilocus enzyme electrophoresis (9). All nine toxigenic strains of *C. argentinense* belonged to one enzyme type based on nine enzyme activities. The three nontoxigenic strains of *C. argentinense* were distinguishable from the toxigenic strains as well as from each other on the basis of enzyme types, but were more closely related to the toxigenic organisms than to any others in the study. The complete uniformity of the toxigenic strains was surprising in view of their diverse origins, having been isolated on two different continents. Preliminary results of similar studies on 26 strains of group I and II *C. botulinum* show 23 different

enzyme types based on 13 enzymes (M. Reeves, L. M. McCroskey, and C. L. Hatheway, unpublished data).

The strain of *C. baratii* that produces type F botulinal toxin (115) and caused type F infant botulism (137) and one of the two strains of *C. butyricum* that produce type E botulinal toxin (192) and caused type E infant botulism in Italy (14) were found to correspond to the type strains of their respective species by DNA analysis, with 86 and 78% relatedness, respectively (312).

Bacteriophages and plasmids and their roles in coding for botulinal neurotoxins. Bacteriophages have been found in studies with groups I, II, and III of *C. botulinum* (75, 87, 142). An association of phages with toxigenicity has been found only with group III. Type C and D strains cured of their phages no longer produce toxin (86). These nontoxigenic organisms can be converted to toxigenic forms, producing either type C or type D toxin, by reinfecting the organisms with a phage isolated from the corresponding toxigenic strain. An interspecies conversion was accomplished by infecting a strain of type C *C. botulinum* cured of its phage with a phage (NA1) derived from *C. novyi*. The reinfected organism was indistinguishable from *C. novyi* type A (88); it then produced the alpha-toxin of *C. novyi* as its dominant toxin (90). These phage studies suggest that *C. botulinum* type C-beta is derived from C-alpha when the latter loses the phage responsible for producing C_1 toxin (149).

Strom et al. (311) isolated plasmids from all four groups of botulinal toxin-producing organisms, including all toxin types. Multiple plasmids were found in strains of group I, types A and B, and group III, types C and D. Single plasmids that differed between strains were found in group II strains of types B and E. A single uniform plasmid was found in all five strains of group I type F (11.5 megadaltons [MDa]), all four strains of group II type F (2.2 MDa), and all six toxigenic strains of *C. argentinense* (81 MDa). No phenotypic functions were assessed for the plasmids at the time of the study. Subsequently, Eklund et al. (89) found a correlation between the presence of the 81-MDa plasmid in *C. argentinense* and production of type G botulinal toxin as well as production of a bacteriocin. Isolates that became nontoxigenic after growth at 44°C were consistently found to lack the plasmid. After growth at 44°C, all toxigenic isolates had retained their plasmid. In a study of 12 strains of *C. botulinum* type A with differing plasmid profiles, Weickert et al. (340) observed toxigenicity in organisms that originally lacked plasmids, as well as in organisms whose plasmids had been eliminated after growth in media containing sodium deoxycholate. Although toxigenicity was lost by some of the isolates after treatment, the loss was not correlated with plasmid loss. Thus far, the only correlation between botulinal neurotoxin production and the presence of a plasmid has been with type G.

Botulinal Neurotoxins

Botulinal neurotoxins exist in the cultures as macromolecular complexes known as progenitor toxins (274), consisting of a neurotoxin subunit and one or more nontoxic subunits (Fig. 1). For toxin types A, B, C, D (274), and G (227), the nontoxic components of the larger complexes (19S and 16S) may include a hemagglutinin. The larger complexes have not been found for type E and F toxins. The progenitor toxins exist as LL (extra large; 19S; observed only with type A toxin), L (large; 16S), and M (medium; 12S) forms. The toxic component, known as derivative toxin, can be dissociated

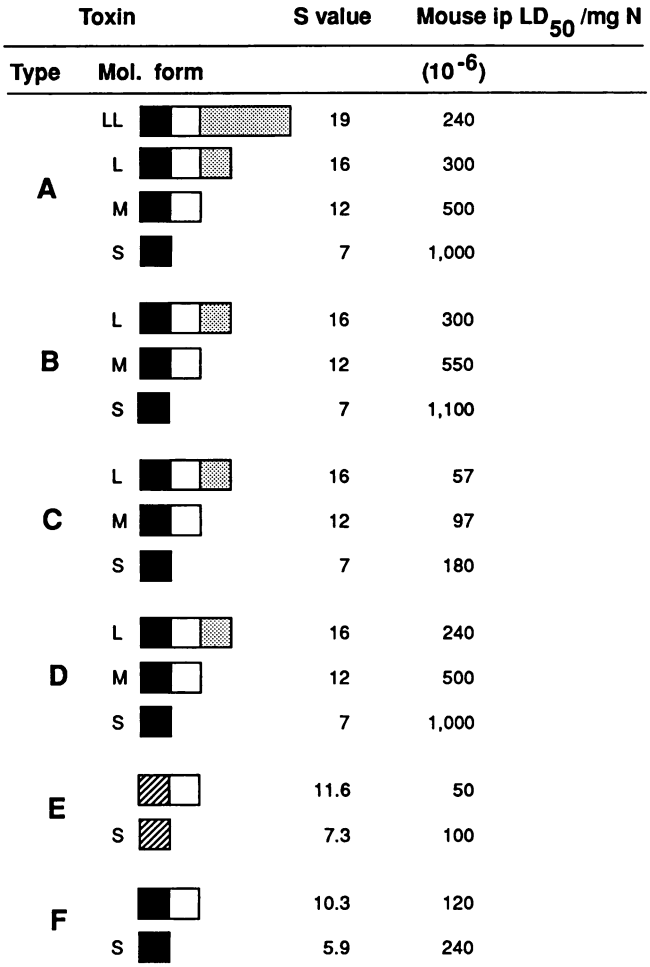

FIG. 1. Molecular sizes, structures, and toxicities of *C. botulinum* progenitor and derivative toxins. Symbols: ■, fully toxic component, constituting each derivative toxin; □, nontoxic; ▨, nonactivated; ⊡, hemagglutinin active. i.p., intraperitoneal; LD_{50}, 50% lethal dose; LL, extra large; L, large; M, medium; S, small. From reference 274, with permission of the author and Pergamon Press, Inc.

and separated from the nontoxic components by DEAE chromatography at a slightly alkaline pH, above 7.2 (274). Derivative toxins have a sedimentation value of 7S, corresponding to a molecular size of about 150 kDa. The progenitor toxin complex appears to be important for oral toxicity, because the purified neurotoxin (derivative toxin) that is so highly active by the parenteral route seems to be inactivated by passage through the stomach (240).

Regardless of serological type, the derivative toxins are very similar to each other and closely resemble tetanus toxin (tetanospasmin) in molecular structure. Several reviews have detailed the molecular aspects of these toxins (112, 274, 292, 315). The purified active derivative toxin consists of a heavy chain (ca. 100 kDa) and a light chain (ca. 50 kDa) held together by a disulfide bridge (Fig. 2). The toxin is initially synthesized as a single chain that is cleaved by a proteolytic enzyme, whether endogenous or exogenous, to form the dichain active structure. Type A toxin is recovered from cultures essentially in the two-chain form; type E, in the inactive single-chain form; and type B, as a mixture, mostly in the inactive single-chain form (280). The heavy chain of

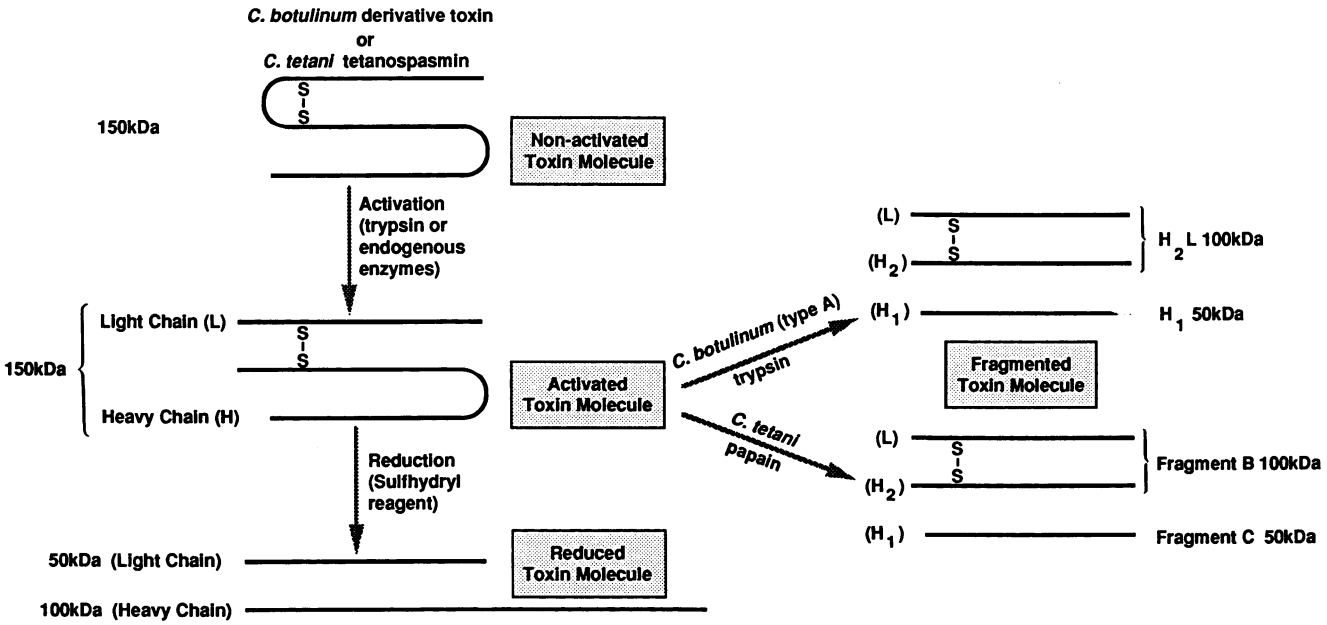

FIG. 2. Diagrammatic representation of *C. botulinum* (derivative) and *C. tetani* neurotoxin structure; activation, fragmentation, and separation of component peptide chains.

the active derivative toxins is subject to cleavage by proteolytic enzymes (161, 287). Trypsin cleaves the heavy chain (H) into two fragments, H_1 (46 kDa), the carboxy end, and H_2 (49 kDa), the amino end that remains attached to the light chain (L) (287). The linked fragment (H_2L) has a molecular mass of 105 kDa. Neither H_1 nor H_2L is toxic, but H_1 appears to possess the binding site because it competes with whole derivative toxin for binding to synaptosomes. H_2L does not bind. These fragments are analogous to the papain fragments C and B, respectively, of tetanus toxin (112) (Fig. 2).

Amino acid analyses of the various types of botulinal toxins (65–68) and of their separated light and heavy chains (279) have been reported. Thus far, only partial amino sequences of the botulinal neurotoxins have been determined (279, 280, 283, 284). Considerable amino acid sequence homology is noted for the known sequences of type A, B, and E light and heavy chains (64, 280). Striking homology has also been observed between known segments of the botulinal toxin chains and segments of the tetanus toxin chains (64, 82). Complete resolution of the botulinal toxins will no doubt be achieved only after the structural genes are isolated and sequenced, allowing researchers to determine the amino acids from the nucleotides coding for them.

Mode of action. Botulism paralysis appears to be a three-step process: (i) recognition of a receptor on the nerve ending and binding of the toxin molecule at that site; (ii) internalization of a portion of the molecule into the nerve cell; (iii) action of the internalized toxin fragment to prevent acetylcholine release from the nerve ending (289, 292). Tetanus toxin shows actions similar to the botulinal toxins in comparable experimental studies with nerve preparations, but higher toxin concentrations are required. The heavy chain is believed to possess the binding site that recognizes the receptor, which may be a ganglioside (112). Bigalke et al. (30) found that neuraminidase treatment of mouse spinal cord cultures diminished the potency of botulinal toxin type A but not of tetanus toxin to block synaptic transmission. Evidence shows that the different types of botulinal toxins bind to separate receptors (112, 274). The binding site of the toxin is associated with the carboxy terminus of the heavy chain, fragment H_1, the portion that is released from the dichain by trypsin cleavage (Fig. 2). The amino terminus of the heavy chain is believed to create channels in the membrane through which the light chain may enter the cell (136, 292). The mechanism by which the internalized toxin fragment interferes with neurotransmitter release is not yet known. A different mode of intracellular action may exist for type A and B toxins. Type B (and perhaps some of the other types) appears to share a mechanism of action more in common with tetanus toxin than with type A toxin. Their effects are not reversed by aminopyridines as are those of type A (96, 288). Gansel et al. (96) found additive poisoning effects on mouse motor nerve terminals when type A toxin was supplied in addition to either type B or tetanus toxin. The latter two were not supplemental to each other.

Medicinal use. Type A botulinal neurotoxin has recently been used successfully for treatment of several neuromuscular disorders (285). It is currently being used for some ophthalmological conditions such as strabismus and blepharospasm, as well as in focal distonia and hemifacial spasm (46), spastic dysphonia (177), and spasmodic torticollis (328). Precisely located injections of minute amounts of the neurotoxin are used to block muscular response to the nerve impulses responsible for the disorders.

Other Toxins Produced by *C. botulinum*

C_2 toxin. After the discovery of *C. botulinum* type C, the distinction of C-alpha and C-beta was established to explain the differential neutralization of toxins of the Bengtson and Sedden strains (108). Jansen (147, 148) determined that C-alpha strains produced a major amount of neurotoxin designated C_1 as well as a minor amount of a second toxin designated C_2. C-beta strains produced only C_2 toxin. C-alpha was also found to produce a minor amount of type D neurotoxin, while type D strains produced a major amount of

type D neurotoxin and a minor amount of C_1. C_2 toxin could be detected in cultures of C-alpha and D strains after trypsin treatment (85). Although initially there was confusion on the matter, it became apparent that C_2 toxin was not a neurotoxin; it was shown to have vascular permeability and lethal activities but no paralytic effect (150, 239, 290). C_2 toxin was found to consist of two separate peptide molecules (144). Component I (C_2I), the lighter molecule with a molecular mass of 50 kDa, and component II (C_2II), with a molecular mass of 105 kDa, are both required for biological activity (238). C_2 toxin is thus referred to as a binary toxin. Vascular permeability can be demonstrated by intradermal (i.d.) injection of both trypsinized components followed by intravenous (i.v.) injection of Evans blue (237, 239). Bluing at the injection site demonstrates the permease activity. The reaction also occurs at the injection site when component II is injected i.d. and component I is injected i.v., but not vice versa. C_2II possesses the binding site and thus localizes the reaction; when C_2I arrives at the site where component II is fixed, the reaction takes place. C_2 toxin also has enterotoxin activity, causing a marked distension of ligated intestinal loops in mice 4 to 8 h after injection (236).

C_2I is an enzyme with ADP-ribosylating activity (291), an activity already known for other bacterial toxins such as diphtheria toxin, cholera toxin, and *Pseudomonas* exotoxin A (10). It ADP-ribosylates a protein of 43-kDa molecular mass obtained from homogenates of mouse or chicken tissues and also an artificial substrate, poly-L-arginine. The reaction takes place when purified nonmuscle actin is used as the substrate in cell-free systems (3, 241). The reaction has no effect on protein synthesis of the cell or on adenylcyclase, with which ADP-ribosylating activity of some other bacterial toxins have been associated (10). It is postulated that C_2II recognizes the receptor on the cell surface membrane and effects the internalization of component I which ADP-ribosylates intracellular actin, reducing the ability of the microfilament protein to polymerize. This explains the observed cellular damage (241).

Exoenzyme C_3. Some investigators have reported that type C and D neurotoxins possess ADP-ribosylating activity, which would be the first enzymatic function to be established for any of the clostridial neurotoxins (15, 186, 234, 235). This activity was convincingly shown to be due to botulinum ADP-ribosyltransferase C_3 (4), an enzyme that was no doubt a contaminant of the C_1 and D toxin preparations (266). C_3 has a molecular mass of about 25 kDa and is distinct from C_1 (150 kDa), C_2I (50 kDa), and C_2II (100 kDa). It catalyzes the ADP-ribosylation of a 21- to 24-kDa GTP-binding membrane protein found in various eucaryotic cells, including platelets, neuroblastoma-glioma hybrid cells, leukocytes, and sperm (4). The substrate is distinct from the 19-kDa GTP-binding molecule involved in the ADP-ribosylation by cholera toxin (326) and cannot be confused with the substrate for C_2, which is actin. C_3 has no effect on intact cells. Enzymatic activity is demonstrated in assays with supernatants of tissue and cell homogenates and purified substrates. Introduction of C_3 into cells by an osmotic shock method resulted in intracellular ADP-ribosylation of the protein substrate and morphological changes in the cells (272).

CLOSTRIDIUM TETANI

Tetanus

Tetanus has been recognized since ancient times and was clearly described by Hippocrates (1). One of the first to hypothesize an infectious agent as its cause was Pirogov, in 1867 (50). In 1884, it was shown that tetanus could be transmitted from a human suffering from the disease to a rabbit by injecting the animal with material from the patient's infected wound; implantation of soil samples into mice, rabbits, and guinea pigs caused symptoms of tetanus in the animals (159). A bacilluslike organism was seen under the microscope in smears from the wounds of patients; drumstick forms of *C. tetani* were first described by Fluegge and by Rosenbach, and a pure culture of the toxigenic organism was obtained by Kitasato (50) by using differential heating. Behring and Kitasato demonstrated that animals could be immunized with tetanus toxin modified by iodine trichloride and that the animals' sera contained neutralizing antibodies (346).

Tetanus is a generalized or localized "hypertonia of the striated musculature frequently accompanied by clonic paroxysmal muscular spasms or contractions" (338). Localized tetanus involves muscle rigidity and spasms near the site of the infected wound. This usually progresses to a generalized tetanus, affecting the entire body. Usually the initial manifestation of tetanus is generalized. Neonatal tetanus, which is quite prevalent in certain areas of the world, is due to infection of the umbilical stump. The severity of tetanus can be classified as (i) mild, with good response to treatment with sedatives and muscle-relaxing drugs and a very low mortality rate; (ii) severe, with moderate response to drugs and a 20 to 40% fatality rate; and (iii) very severe, with poor response to treatment and a 50 to 90% fatality rate (338). Neonatal tetanus is almost always very severe and highly fatal. The incidence of tetanus in the United States between 1979 and 1986 has ranged between about 60 and 95 cases per year (58). In 1987, 48 cases were reported, none of them in children <1 year old.

Tetanus toxin acts by blocking inhibitory synapses of the spinal cord motoneurons (196). The toxin prevents release of the inhibitory mediators gamma-aminobutyric acid and glycine, allowing uncontrolled stimulation of the muscles. Toxin spreads from the infected site (i) by diffusing into the adjacent muscle tissues, (ii) by transport by the lymphatic system, or (iii) by passage through the nerves. Toxin enters the blood from the lymphatic system. The toxin molecule attaches to a receptor on the nerve ending, and a fragment of the bound toxin is taken into the nerve cell and passes on to the central nervous system by retrograde movement through the nerve axons.

The Organism

C. tetani is a strict anaerobe and will grow on the surface of agar media only in an anaerobic environment such as in an anaerobic chamber with an atmosphere of 85% nitrogen–10% hydrogen–5% carbon dioxide. The bacilli are 0.5 to 1.7 by 2.1 to 18.1 μm and often possess terminal endospores that give them a "drumstick" appearance. Cells in cultures older than 24 h may appear gram negative. They are motile by means of peritrichous flagella. Optimal growth occurs at 37°C, while little or no growth takes place at 25 or 42°C. Growth may not be apparent until after 48 h and may appear as a film rather than as discrete colonies because of swarming due to the vigorous motility. Media containing 3 to 4% agar are more conducive to formation of discrete colonies. On blood agar, the colonies are 4 to 6 mm in diameter, flat, translucent, and gray with a matte surface, showing a narrow zone of clear (beta-type) hemolysis. Colonies have irregular and rhizoid margins (54).

Most common biochemical tests are negative. No sugars are fermented; milk and other complex proteins are not digested. Neither lecithinase nor lipase is produced, and nitrate is not reduced. Gelatin is liquefied slowly, requiring perhaps 7 days for complete liquefaction. Hydrogen sulfide and indole are produced. Gas-liquid chromatography of peptone-yeast extract broth cultures shows butanol and acetic, propionic, and butyric acids as metabolic end products (139). Spores generally survive moderate heating (75 to 80°C for 10 min) but usually are destroyed after exposure to 100°C for 1 h (1).

C. tetani is similar culturally and biochemically to *C. cochlearium* and organisms identified as *C. tetanomorphum* (the latter is no longer listed in *Bergey's Manual* [54]), but all three groups could be clearly distinguished on the basis of DNA homology (216). Nontoxigenic strains of *C. tetani* are found that correlate well with toxigenic strains by DNA comparison. Soluble cellular proteins of *C. tetani* and *C. cochlearium* may be distinct (53), but otherwise it seems difficult to distinguish between the latter and nontoxigenic *C. tetani* without DNA studies.

C. tetani is commonly found in soil samples in all parts of the world. Surveys in Japan, Canada, Brazil, and the United States have yielded 30 to 42% positive samples (298). It is also commonly found in human and animal feces. Bauer and Meyer (20), Ten Broeck and Bauer (322), and Tulloch (329) found the organism in about 25 to 35% of human fecal specimens in the United States, China, and England. However, many of the more recent studies on human fecal flora failed to find *C. tetani* (98).

Laboratory confirmation of tetanus. Tetanus can be confirmed bacteriologically by isolating *C. tetani* from the infected wound. This is not usually done, however, because the disease is so easily diagnosed clinically on the basis of its unique, recognizable, classic signs. Therefore, very few isolates of the organism from clinical cases have been studied. Information on clinical isolates could be valuable, for example, if serological variants of the toxin were detected that might account for occurrence of tetanus in immunized patients with circulating antitoxin (24, 244). Toxicity and neutralization tests can be performed by intramuscular injection of toxic supernatant or whole culture mixed with 2% calcium chloride into untreated mice and mice protected with tetanus antitoxin (21).

The Toxin (Tetanospasmin)

Tetanus toxin is produced in the bacterial cell as a single polypeptide chain with a molecular mass of approximately 150 kDa (31, 112). After release from the cell, the molecule is cleaved into two polypeptide fragments: a heavy chain of about 100 kDa and a light chain of about 50 kDa that remain joined by a disulfide bond. The cleavage or "nicking" is caused by proteases produced by the organism (132). The macromolecular structure of the toxin is remarkably similar to that of botulinal (derivative) toxin and is represented diagrammatically in Fig. 2. Treatment of the toxin with a reducing agent such as dithiothreitol eliminates the disulfide bridge and dissociates the heavy chain and light chain. Papain treatment of the intact bichain toxin cleaves the heavy chain, resulting in two fragments: fragment B, a bichain molecule with a mass of 95 kDa, consisting of the light chain and the shortened heavy chain; and fragment C derived from the heavy chain with a mass of 45 kDa (132). These fragments are nontoxic, as are the separated heavy and light chains. The fragments and heavy and light chains retain their serological characteristics, and all react with antiserum to the whole toxin molecule (133). In immunodiffusion analysis, the heavy and light chains show no precipitation lines of identity with each other. Lines of partial identity were found between fragment B and each of the separated chains and between fragment C and the heavy chain. No cross-reactivity exists between fragment C and either fragment B or the light chain. These serological results are consistent with the molecular structure represented in Fig. 2. The portion of the molecule that binds to receptors on the nerve endings in the initial step in the paralytic process appears to be on the heavy chain and on fragment C (208). The affinity of (i) the toxin molecule, (ii) the heavy chain, and (iii) fragment C for gangliosides suggests involvement of ganglioside structures in the membrane receptors for the toxin (208, 334). A binding system involving a second receptor, a protein, has been proposed to account for inadequacies of gangliosides alone serving as the receptors (204).

Plasmids and the toxin gene. Studies on bacteriophages of *C. tetani* failed to establish any relationship between a bacteriophage and toxigenicity of the organism (116, 257, 258). Laird et al. (162) found that toxigenicity was correlated with the presence of a single large plasmid. Subsequent studies with oligonucleotides coding for an amino-terminal amino acid sequence as gene probes showed that the gene is on the plasmid (93). Eisel et al. (82) isolated a 75-kilobase (kb) plasmid from the Massachusetts strain of *C. tetani* and established that it contained the toxin gene, using gene probes coding for six amino acids near the amino terminus of fragment C. The plasmid was characterized by partial digestion with endonucleases. Seven fragments of the plasmid with overlapping sequences were cloned in *Escherichia coli*, and the DNA sequence of each was determined. From the resulting data, the entire 3,945-nucleotide sequence of the toxin gene was determined, and the correct 1,315-amino-acid sequence of the toxin molecule could readily be deciphered. The structure has a calculated molecular weight of 150,700. Fairweather and Lyness (92) reported sequences identical to those of Eisel et al. (82) for the nucleotides of the gene and the amino acids of the tetanus toxin.

Other Toxins: Tetanolysin

C. tetani produces an oxygen-sensitive hemolysin known as tetanolysin that is related functionally and serologically to streptolysin O and to the oxygen-sensitive hemolysins of a variety of other organisms, including at least six species of *Clostridium* (26). Purified tetanolysin has a molecular mass of 48 ± 3 kDa and appears to have several molecular species based on ionic change, exhibiting pIs of 5.3, 5.6, 6.1, and 6.6 (176, 202). Some of the isoelectric point variation may be due to differences in charge between oxidized and reduced forms of the same molecule (61). This group of hemolysins lyses a variety of cells such as erythrocytes, polymorphonuclear leukocytes, macrophages, fibroblasts, ascites tumor cells, HeLa cells, and platelets (26). Lytic activity is reversibly inactivated by mild oxidizing conditions such as aeration or hydrogen peroxide and reactivated by a thiol reagent such as cysteine, reduced glutathione, or thioglycolate. The active toxin has an affinity for cholesterol and related sterols, and its lytic and lethal activities are inhibited by these compounds. Hardegree et al. (117) showed in vivo effects of tetanolysin after i.v. injection into animals. In mice, pulmonary edema rapidly developed, followed by death. In rabbits and monkeys, evidence showed intravascular hemolysis.

TABLE 4. Toxin types of *C. perfringens* and major and minor toxins they produce

Toxins	Biological activity	Toxin type[a]				
		A	B	C	D	E
Major lethal						
Alpha	Lethal, lecithinase (phospholipase C)	+	+	+	+	+
Beta	Lethal, necrotizing, trypsin labile	−	+	+	−	−
Epsilon	Lethal, permease, trypsin activatable	−	+	−	+	−
Iota	Lethal, dermonecrotic, binary, ADP-ribosylating, trypsin activatable	−	−	−	−	+
Minor						
Gamma	Not defined, existence questionable	−	±	±	−	−
Delta	Hemolysin	−	±	+	−	−
Eta	Not defined, existence questionable	±	−	−	−	−
Theta	Hemolysin (O_2 labile), cytolysin	±	+	+	+	+
Kappa	Collagenase, gelatinase	+	+	+	+	+
Lambda	Protease	−	+	−	+	+
Mu	Hyaluronidase	±	+	±	±	±
Nu	DNase	±	+	+	±	±
Neuraminidase	*N*-Acetylneuraminic acid glycohydrolase	+	+	+	+	+
Other						
Enterotoxin	Enterotoxic, cytotoxic	+	NT	+	+	NT

[a] +, Produced by most strains; ±, produced by some of the strains; −, no strain of the indicated toxin type has been shown to produce the toxin. NT, Insufficient studies have been done with strains of the indicated toxin type.

Monkeys showed electrocardiographic changes and a decreased heart rate, indicating a cardiotoxic effect of tetanolysin. It is questionable whether this toxin plays any role in *C. tetani* infections. The amount of tetanolysin produced by the organism in vivo is unknown, and serum that contains cholesterol is known to inhibit its activity in vitro.

CLOSTRIDIUM PERFRINGENS

C. perfringens is the most widely occurring pathogenic bacterium (298). It is readily found in soil samples and intestinal contents of animals and humans. The organism, first described by Welch and Nuttall in 1892 as *Bacillus aerogenes capsulatus* (342), has also been commonly known as *C. welchii*, especially in Great Britain. Spores of *C. perfringens* usually are rare or not observed in cultures grown in ordinary media, so it must be recognized by its other characteristics (139). It grows vigorously at temperatures between 20 and 50°C, with an optimum of 45°C for most strains (54). Colonies on blood agar usually show a characteristic double-zone hemolysis around the colony: an inner clear zone due to theta-toxin; and an outer hazy zone due to alpha-toxin. On McClung-Toabe egg yolk agar, the colonies are surrounded by a wide circular opaque zone, recognized as the lecithinase reaction due to the alpha-toxin (54). The organisms are gram-positive straight rods with blunt ends that occur singly or in pairs, 0.6 to 2.4 μm wide by 1.3 to 19.0 μm long. They are nonmotile, reduce nitrate, ferment glucose, lactose, maltose, sucrose, and other sugars, and liquefy gelatin. "Stormy fermentation" in milk is seen, due to the fermentation of lactose, production of gas, and clotting, but not digestion of casein. The organism is usually easy to recover from mixed cultures incubated at 45°C (its optimal temperature for growth) because of its short generation time (as short as 8 min) (298), which allows it to outgrow other organisms present.

McDonel has provided a thorough review of all of the major and minor toxins produced by *C. perfringens* (193). He lists the four major lethal toxins (alpha, beta, epsilon, and iota) on which the toxin types of the species are based; nine minor toxins (or soluble antigens) that may or may not play some role in pathogenicity (delta, theta, kappa, lambda, mu, nu, gamma, eta, and neuraminidase); and enterotoxin, which is responsible for *C. perfringens* foodborne illness.

Toxin Types of *C. perfringens*: Diseases

Five toxin types of *C. perfringens* are now recognized on the basis of which lethal toxins (alpha, beta, epsilon, and iota) are produced. In 1931, Wilsdon (cited in reference 102) established four toxin types, A, B, C, and D, of *C. perfringens* by preparing toxic culture filtrates and their corresponding antitoxic sera and observing the differential mouse protection by the sera. Wilsdon found that type A antiserum neutralized only type A cultures, type B neutralized all four types, type C neutralized all but type D, and type D neutralized both type A and D cultures. He recognized that his immunizing filtrates contained multiple antigens. These antigens were later designated as alpha, present in all four types; beta, present in types B and C; and epsilon, present in types B and D (306). A fifth type (type E) was established by Bosworth in 1943 when he isolated a strain from a calf that produced a lethal toxin that could not be neutralized by type A, B, C, or D antitoxin or any combination thereof (44). Type E antitoxin neutralized type A toxic filtrates as well as those of the homologous strain but not those of type B, C, or D. Bosworth proposed that the unique lethal factor in type E cultures be designated as iota. The toxin types are listed in Table 4 with the major lethal toxins by which they are identified and the minor toxins that they produce. Type F had been established for a strain isolated from an outbreak of human necrotic enteritis in Germany (355). The organism produced alpha- and beta-toxins and would have been classified as type C on that basis, but was unusually heat resistant and lacked three minor antigens (delta, theta, and kappa) found with the classic type C strains. This distinction was subsequently deemed unjustified because discrepancies in ancillary characteristics among other strains were noted, and establishing new types to denote such differences would become unmanageable (306). Originally, the lethal toxins

TABLE 5. Diseases caused by *C. perfringens* toxin types A, B, C, D, and E

Toxin type	Diseases
A	Gas gangrene (myonecrosis), foodborne illness, and infectious diarrhea in humans; enterotoxemia of lambs, cattle, goats, horses, dogs, alpacas, and others; necrotic enteritis in fowl; equine intestinal clostridiosis; acute gastric dilation in nonhuman primates, various animal species, and humans
B	Lamb dysentery; sheep and goat enterotoxemia (Europe, Middle East); guinea pig enterotoxemia
C	Darmbrand (Germany) and pig-bel (New Guinea) in humans; "struck" in sheep; enterotoxemia in lambs and pigs; necrotic enteritis in fowl.
D	Enterotoxemia of sheep; pulpy kidney disease of lambs
E	Enterotoxemia in calves; lamb dysentery; guinea pig enterotoxemia; rabbit "iota" enterotoxemia

were assayed by i.v. inoculation of mice. Routine toxin typing of *C. perfringens* (*C. welchii*) was later performed by a combination of serological tests for egg yolk reaction (alpha-toxin), hemolysis of sheep erythrocytes (delta- and theta-toxins), azocoll reaction (kappa- and lambda-toxins), and i.d. injection into guinea pigs (beta- and epsilon-toxins) (231). Current recommendations (77, 139) involve intraperitoneal injection into mice of supernatant fluid from 4- to 6-h cultures in chopped-meat–glucose–starch medium. Both untreated and trypsinized samples are tested, with and without each of the typing sera. Trypsin is necessary to activate both the epsilon- and iota-toxins, if present, but since trypsin inactivates beta-toxin, both treated and untreated samples must be tested. Not all strains of *C. perfringens* type A produce sufficient alpha-toxin under these conditions to kill mice. Thus, strains which are nonlethal for mice are considered as type A strains by some researchers (139).

The diseases caused by the different toxin types of *C. perfringens* are listed in Table 5. *C. perfringens* type A is commonly found in soil samples, indeed, in almost every sample that has been specifically cultured for it (298). This organism is also readily found in intestinal contents of humans and other animals in the absence of disease. Type A strains are often responsible for gas gangrene (myonecrosis) (174, 184) and foodborne illness in humans (310). A newly described clinical entity, *C. perfringens* enterotoxin-associated (38, 43) or infectious (164) diarrhea, appears to be due to antibiotic-associated intestinal colonization of enterotoxin-producing type A strains. Type B, C, D, and E strains do not survive in soils. They seem to be obligate parasites. They are found in the intestinal tract of domestic animals and occasionally humans usually associated with disease. Strains of *C. perfringens* that produce beta-toxin generally are of more concern for animal than for human health. Type B strains have been implicated in lamb dysentery, and in sheep and goat enterotoxemia in Europe and the Middle East, but have not been encountered in North America or Australia. Type C strains have caused severe necrotic enteritis in humans due to the beta-toxin: "Darmbrand" in Germany (355) and "pig-bel" in New Guinea (213). They are the cause of "struck" in lambs and sheep in Europe, and they have been encountered in necrotic enteritis in cattle, lambs, and pigs in the United States (193). These and other diseases of animals caused by strains of types A, B, C, D, and E are listed in Table 5.

C. perfringens Foodborne Illness

The earliest report of foodborne illness outbreaks due to *C. perfringens* was provided by McClung in 1945 (189). In four episodes, patients experienced nausea, intestinal cramps, and a pronounced diarrhea 8 to 12 h after consuming chicken dishes prepared on one day and served the following day. Samples of the foods showed heavy contamination with gram-positive rods, which were identified as *C. perfringens*. Hobbs et al. (135) observed similar outbreaks in England; the causative organisms were of toxin type A. They noted that the spores of implicated strains were considerably more heat resistant and the organisms were less hemolytic than the classic strains. Outbreak strains in the United States were usually more like the classic strains, with spore death within 10 min at 100°C, rather than spore resistance to 100°C for more than 1 h as observed with the British strains. Sutton and Hobbs (317) later found that both heat-susceptible and heat-resistant strains were implicated in England. Symptoms of the illness occurred in human volunteers after ingestion of a large number of viable vegetative bacteria (more than 5×10^8), but not after ingestion of spores or cell-free culture fluid (73, 127). An enterotoxin released from the cells during sporulation was responsible for the diarrheal illness (79, 80). It has been generally assumed that enterotoxin is a by-product of sporulation, perhaps a spore coat protein, but synthesis of substantial amounts of enterotoxin by non-sporulating cultures has been demonstrated convincingly (103).

C. perfringens foodborne illness undoubtedly occurs frequently, but because of the mildness of the disease it is often not reported. Between 1970 and 1980, 567 outbreaks of foodborne illness due to *C. perfringens* were confirmed in England (310). In the United States, about 25 outbreaks are reported each year (57, 123).

Laboratory investigation. Criteria for confirming *C. perfringens* foodborne illness include the following: (i) count of $>10^5$ *C. perfringens* per g of suspected food; (ii) median spore count of $>10^6$ *C. perfringens* per g of stools from group of affected patients; or (iii) isolation of *C. perfringens* of a common serotype from stools from groups of affected patients and from suspected food (124). Serological tests for detecting enterotoxin in feces of patients are now available (27, 28). The reversed passive latex agglutination test is commercially available, and in the United States it can be obtained from Oxoid USA, Columbia, Md. It appears to be suitable for use in diagnostic laboratories (119). Serotyping of organisms has been used successfully for laboratory confirmation of *C. perfringens* foodborne outbreaks in England (310). It has been of limited value in the United States (123), because serotyping has been carried out there by using an initial bank of reagents without supplementing with additional reagents necessary to identify untypable strains encountered subsequently.

Enterotoxin-Associated Diarrhea

Evidence for *C. perfringens* enterotoxin-induced diarrhea in the absence of a food vehicle containing large numbers of organisms has been accumulating. Larson and Borriello (164) reported 50 cases of *C. perfringens* diarrhea with no association with food poisoning. Diarrhea was associated with antibiotic treatment and involved, almost exclusively,

elderly patients (38, 43, 164). *C. perfringens* numbered from 5×10^7 to 4×10^9 per g of stool obtained in the acute phase, and enterotoxin titers ranged from 1:4 to 1:5,120. No *C. difficile* toxin was detected in any of the fecal specimens. The cases occurred in hospitals and were sporadic, not suggestive of a foodborne source. The diarrhea was prolonged, with median duration of 7 days, and relapses occurred in some cases. In foodborne diarrhea, symptoms usually subside within 24 h.

Major Lethal Toxins

Alpha-toxin. Alpha-toxin, produced in large amounts by type A strains, is a phospholipase C (EC 3.1.4.3) and is believed to be the major factor responsible for the tissue pathology in myonecrosis (gas gangrene) caused by this organism. Immunization of guinea pigs with alpha-toxin protects them against gas gangrene when challenged with *C. perfringens* and toxin (153). Alpha-toxin has been shown to affect myocardial function, causing hypotension and bradycardia, resulting in shock, a common and often fatal feature of gas gangrene (307). The toxin also increases vascular permeability, as can be demonstrated by i.d. injection (314). The toxin hydrolyzes phosphatidylcholine and, to a lesser extent, sphingomyelin, but not other phospholipids (151). The active enzyme is a zinc metal protein and requires calcium ions for interaction with substrate. It is responsible for the lecithinase reaction on egg yolk medium and for the hazy zone of hemolysis on blood agar.

Purified alpha-toxin has a molecular mass of 43 kDa and an isoelectric pH of 5.4 (151). Recently, three laboratories almost simultaneously reported cloning the alpha-toxin gene from *C. perfringens*. Titball et al. in England (323) incorporated fragments of the *C. perfringens* genome into an *E. coli* plasmid and detected organisms carrying the recombinant plasmid containing the gene by their lecithinase reaction on egg yolk agar plates. The gene coded for a protein that had a molecular mass of 44.5 kDa and appeared to be identical to the alpha-toxin produced by the donor strain. Tso and Seibel in the United States (327) cloned the gene by similar methods. Alpha-toxin production of plasmid-bearing organisms was detected by the hemolytic reaction on blood agar plates. They found a 2-kb fragment inserted into the plasmid that contained a 1,197-nucleotide sequence from *C. perfringens* that coded for the 399-amino-acid peptide with a molecular mass of 43 kDa. The peptide had biological activities similar, if not identical, to those of the alpha-toxin of the donor strain. These researchers also cloned the gene for *C. bifermentans* phospholipase C and found it to be 64% homologous in coding sequence with the *C. perfringens* alpha-toxin gene. The gene product had 1/50 the activity of the native *C. perfringens* product and of the cloned *C. perfringens* gene product as measured by hemolysis or by phosphatidylcholine hydrolysis. Okabe et al. in Japan (242) also successfully cloned the *C. perfringens* alpha-toxin gene and have published nucleotide and amino acid sequences identical to those of the other two studies.

In foodborne disease outbreaks due to *C. perfringens*, detection of alpha-toxin in food has been used to determine the source (70, 118). The hemolytic and lecithinase activities of the alpha-toxin can be used as an index of bacterial growth.

Beta-toxin. Beta-toxin is a major lethal toxin produced by both type B and C strains of *C. perfringens* (Table 4). It is responsible for the lesions of necrotic enteritis of pig-bel (213) and Darmbrand (355). Lawrence and Cook (168) showed that beta-toxin of type C organisms will produce necrotic lesions in the small intestines of guinea pigs only when protease inhibitor is introduced into the gut. The lesions in the guinea pigs were similar to those observed in human pig-bel patients. The experimental results supported the following hypothesis: that pig-bel occurs because the victims have low protease activity in their intestines due to a normally low protein diet and that the low level of protease activity is further inhibited by protease inhibitors present in sweet potatoes that are consumed in large quantities at the New Guinea feasts, along with the pork that serves as the source of the pathogen (213). Immunization of guinea pigs with beta-toxin protects them from the pig-bel-like disease after challenge with either toxin or organism and the protease inhibitors.

Beta-toxin can be recovered from culture fluid and purified by affinity chromatography, using a column containing chelated zinc, and a second chromatography on hydrophilic vinyl resin (276). The purified toxin has a molecular mass of 40 kDa and an isoelectric pH of 5.6. These properties are similar to those of alpha-toxin, and for this reason it is difficult to separate the two toxins. Administered i.v. into rats, beta-toxin causes a rise in blood pressure and a fall in heart rate. The toxin apparently induces the release of catecholamines that are responsible for the increased blood pressure (277, 278). When injected i.d. into guinea pigs, a minimum dose of 2 ng causes purple areas of dermonecrosis. The 50% lethal doses for adult mice are 310 and 4,500 ng/kg i.v. and intraperitoneally, respectively (276).

Epsilon-toxin. Both type B and D strains of *C. perfringens* produce epsilon-toxin. It is produced as a prototoxin that is activated by proteolytic enzymes produced by the same organism (the kappa- and lambda-toxins) (345), or it can be activated by adding trypsin to the culture. The prototoxin is a protein consisting of 311 amino acids, with a molecular mass of 34.25 kDa based on sedimentation coefficient (111). Activation of the prototoxin with trypsin involves breaking of the peptide bond between the 14th and 15th amino acids from the amino terminus (Lys-14–Ala-15) and releasing an "activation peptide" of 14 residues (29). Epsilon-toxin primarily affects the intestine by increasing permeability of the gut wall, thus enhancing its own uptake, and acts systematically as a lethal toxin (49). After entering the circulation, it causes swollen hyperemic kidneys, edema in the lungs, and excess pericardial fluid (193). The effect of increasing vascular permeability can be demonstrated by i.d. injection of the toxin, after which the injected site will be permeable to circulating Evans blue (49). Buxton (49) proposed a possible mechanism of action of epsilon-toxin which directly or indirectly involves the adenylcyclase system in affected cells. An enzyme-linked immunosorbent assay for epsilon-toxin has been described and proposed as an alternative to mouse lethality and specific antibody protection tests (220).

Iota-toxin. Toxin type E of *C. perfringens* was established to classify a strain isolated from a calf that had died 12 h after onset of illness with signs of enteritis and general intoxication (44). The unique toxic factor produced by the new strain was designated iota, in accord with the nomenclature system established by Glenny et al. (102). In addition to mouse lethality after i.v. injection, iota-toxin has dermonecrotic activity (44). It was found to be activated by trypsin in cultures incubated for up to 5 to 7 h, but not in older cultures (296). At subnecrotizing doses, iota-toxin injected intracutaneously increases vascular permeability (62).

Recently, it was demonstrated that iota-toxin consists of two separate proteins that are immunologically and bio-

chemically distinct, designated iota-a and iota-b (308, 309). The two components were separated by isoelectric focusing, showing isoelectric pH values of 5.2 and 4.2 and molecular masses of 47.5 and 71.5 kDa, respectively. A mixture of both components is required for significant biological activity as measured by mouse lethality or dermonecrosis. The light chain (iota-a) is an enzyme that ADP-ribosylates polyarginine (294) and skeletal muscle and nonmuscle actin (282, 332). A subtle substrate distinction exists between iota-a and C_2I in that the latter can ADP-ribosylate only nonmuscle actin. Iota-toxin of *C. perfringens* is similar to that of *C. spiroforme* in serological, biological, and enzymatic activities (251). See the discussion of interrelations among the clostridial binary and ADP-ribosylating toxins in the last section of this review.

Enterotoxin

C. perfringens enterotoxin was isolated and purified (126, 275, 304) and found to be a protein with a molecular mass of 35 kDa and an isoelectric pH of 4.3. The amino acid sequence has been determined (104, 264). The toxin consists of one peptide of 309 amino acids with a molecular weight of 34,262. The peptide has one free sulfhydryl group. The activity of the enterotoxin is enhanced threefold by treatment with trypsin (105). Trypsin cleaves at two sites, each involving a lysine residue, cleaving 15 or an additional 10 amino acids from the amino-terminal end of the toxin (263). The trypsinized toxin then consists of 284 amino acids and two short peptides of 10 and 15 amino acids. Evidence shows that the short peptides do not separate from the main molecule in the absence of sodium dodecyl sulfate or other denaturants. A 6.8-kb fragment from *C. perfringens* chromosomal DNA, which includes a translational reading frame for the first 30 amino acids of the enterotoxin molecule, has been cloned (143).

The mechanism of action of the enterotoxin seems to involve direct binding of the toxin to receptors on the surface of intestinal epithelial cells. Wnek and McClane (347) isolated a 50-kDa protein from rabbit intestinal brush border membranes that specifically inhibits cytotoxicity for Vero cells. This molecule may be the specific receptor for the enterotoxin. Binding is followed by insertion of the entire molecule into the cell membrane, but no internalization into the cell (187, 188). A sudden change of ion fluxes occurs, affecting cellular metabolism and macromolecular synthesis. As intracellular calcium ion levels increase, morphological damage occurs, resulting in greatly altered membrane permeability and loss of cellular fluid and ions and moderate-sized molecules up to 3.5 kDa (188). Under some conditions a loss of protein molecules may occur, but this may reflect cell death (187). Based on rabbit studies, the enterotoxin is most active in the ileum, moderately active in the jejunum, and essentially inactive in the duodenum (194). Enterotoxin has been studied almost exclusively in type A strains. It has been established that type C and D strains also can produce enterotoxin (193). Only a few strains of types B and E have been tested for enterotoxin production, and all were negative.

Minor Toxins

The minor toxins produced by *C. perfringens* are listed in Table 4. At least some strains of all toxin types produce theta, kappa, mu, nu, and neuraminidase. Delta-toxin is a hemolysin produced by type B and C strains, but not by type A, D, and E strains. Lambda has not been detected in type A and C strains. It is a proteinase that digests gelatin, hemoglobin, and casein to some extent, but not collagen (298). Strains of any toxin type may be encountered which fail to produce one or more of the indicated minor toxins. The minor toxins have been of primary interest in the early attempts at classifying members of the species. Kappa-toxin (collagenase), mu-toxin (hyaluronidase), and lambda-toxin (protease) appear to play roles in pathogenesis because of their ability to break down host tissues. Gamma- and eta-toxins have been proposed to account for discrepancies in neutralizing results obtained with specific antisera, but specific active substances related to those supposed entities have not yet been isolated (193).

Delta-toxin. Delta-toxin has been recovered from *C. perfringens* type C and purified (7, 324). It has a molecular mass of 42 kDa and an isoelectric pH of 9.1. It has high hemolytic activity for erythrocytes from sheep, goats, and pigs, but is relatively inactive against those from humans, horses, rabbits, mice, and other mammals. The activity is inhibited by gangliosides (especially G_{M2}), but not by other lipid compounds such as sphingomyelin, lecithin, or cholesterol.

Theta-toxin. Theta-toxin, also known as perfringolysin O, is responsible for the clear zone of hemolysis produced by at least some strains of all toxin types (193, 298). It is an oxygen-labile, thiol-activated cytolysin, similar but not identical to some hemolysins produced by other species (*Streptococcus pyogenes*, *Streptococcus pneumoniae*, *C. tetani*, and *C. novyi*) (26, 298). Theta-toxin has been purified by anion-exchange and gel permeation chromatography (352) and has a molecular size of 51 kDa by polyacrylamide gel electrophoretic analysis. This estimate agrees with amino acid analysis. Theta-toxin, activated by cysteine, is lethal for mice (i.v. injection), and the lethality is reportedly due to the cardiodepressant effect of toxin-induced release of endogenous mediators such as platelet-activating factor (307).

Tweten (330, 331) recently has cloned and expressed the gene for theta-toxin in *E. coli*. The gene product was identical to the theta-toxin produced by the donor strain in amino-terminal sequence and in sodium dodecyl sulfate-gel electrophoretic analysis. Both had an estimated molecular mass of 54 kDa and were of comparable hemolytic activity. The 1.8-kb chromosomal fragment generated a peptide of 499 amino acids that included a 27-residue signal peptide. The secreted form of the theta-toxin has a calculated molecular weight of 52,469. The toxin has 65% homology with streptolysin O and 42% with pneumolysin. All three hemolysins share an identical region of 12 amino acid residues that include the single cysteine residue of the molecule, which is involved in the thiol activation.

Neuraminidase. Neuraminidase, or sialidase, serves many microorganisms as a pathogenicity factor in a variety of ways (211). Its action on erythrocytes may render them panagglutinable, resulting in increase of blood viscosity and promoting capillary thrombosis. Its modification of gangliosides on host cell surfaces may allow more direct contact of pathogens with the host, or it can provide suitable receptors for other toxins produced by the same or other microorganisms.

Nees et al. (221) recovered and purified neuraminidase from *C. perfringens* ATCC 10543 and found it to be a single peptide with a molecular mass of about 64 kDa and exhibiting five components on isoelectric focusing, each with a different isoelectric pH, ranging between 4.7 and 5.4. After denaturation with sodium dodecyl sulfate or 8 M urea, those differences disappeared and only a single component with an

TABLE 6. Physical characteristics and biological activities of toxins A and B of *C. difficile*[a] and HT[b] and LT[c] of *C. sordellii*

Toxin	Molecular size (kDa)	Isoelectric pH	Dose (ng)			
			Tissue culture	Enterotoxic	Lethal, mouse	Vascular permeability
C. difficile						
A	440–500	5.2–5.7	10	1,000[d]	50	1–10
B	360–470	4.1–4.5	0.0002–0.001	Negative[d]	50	1–10
C. sordellii						
HT	450–525	6.1	500	2,000[d]	120	
LT	240–250	4.55	16.4	Positive[e]	2.94	

[a] From Lyerly et al. (178).
[b] From Martinez and Wilkins (185).
[c] From Popoff (250).
[d] Rabbit loop.
[e] Guinea pig loop.

isoelectric pH of 4.3 was seen. Rood and Wilkinson (268) recovered three different neuraminidases from strain CN 3870 with molecular masses of 310, 105, and 64 kDa. The two larger enzyme species were associated with hemagglutinin potency, but the 64-kDa species was devoid of that activity. Recently, Roggentin et al. (267) cloned a 2.1-kb *Sau*3A DNA fragment from *C. perfringens* A99 whose product had neuraminidase activity. The active product was a peptide of 382 amino acids with a molecular mass of 42.8 kDa. Thus, different neuraminidase enzymes have been recovered from different *C. perfringens* strains.

CLOSTRIDIUM DIFFICILE

For a comprehensive coverage of *C. difficile*, its disease and toxins, the reader is referred to the review on the subject by Lyerly et al. (178) that appeared recently in this journal.

The Organism

C. difficile was discovered in 1935 as a member of the microflora of the intestine of normal infants (114). The organism is a motile, gram-positive rod, with dimensions of 0.5 to 1.9 by 3.0 to 16.9 μm, which forms oval subterminal spores. *C. difficile* liquefies gelatin but is nonproteolytic with respect to milk and meat proteins. It ferments fructose, glucose, mannitol, mannose, and usually xylose. It is negative for lecithinase and lipase and produces acetic, isobutyric, butyric, isovaleric, valeric, isocaproic, formic, and lactic acids in peptone-yeast extract-glucose (54, 139). This organism may be isolated from fecal specimens, using cycloserine-mannitol agar, cycloserine-mannitol blood agar (76), cycloserine-egg yolk-fructose agar, or cycloserine-cefoxitin-egg yolk-fructose agar (99).

Toxins

C. difficile was not known to be pathogenic until it was implicated as the cause of antibiotic-associated pseudomembranous colitis. A toxin neutralized by *C. sordellii* antitoxin was found in the feces of patients (166, 265), but the organism that produced it was actually *C. difficile* (17, 165). The toxin had both enterotoxic and cytotoxic activities, which were subsequently separated from each other and designated as toxin A and toxin B, respectively (321). Purification of toxins A and B has been done by several researchers. Sullivan et al. (316) separated the two toxins, purified them by DEAE ion-exchange chromatography, and observed molecular masses of 440 to 500 kDa for toxin A and 360 to 470 kDa for toxin B. Banno et al. (16) purified the corresponding toxins (designated D-1 and D-2) to homogeneity as determined by polyacrylamide gel electrophoretic analysis, using gel filtration and ion-exchange chromatography. Their molecular masses were 550 to 600 and 450 to 500 kDa, respectively. Each of the two toxins, D-1 and D-2, was converted to an apparent single smaller molecular form, 190 to 200 kDa, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after heating at 100°C for 5 min in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. The toxins were found to be immunologically differentiable by cross-neutralization studies in cytotoxicity and mouse lethal assays (16, 172). Cross-reactivity without neutralization between the toxins and corresponding heterologous monoclonal antibodies, however, have been observed (81, 271), thus indicating common epitopes.

Toxin A is primarily responsible for the enterotoxic activity of *C. difficile* (325), while toxin B is much more potent as a cytotoxin than toxin A (173, 255, 270). Both toxins are lethal for mice, with toxin A having a potency of 50 to 400 times that of toxin B (16, 321). The physical characteristics and biological activities of the two toxins are compared in Table 6. The data for similar toxins produced by *C. sordellii* are also listed.

The enterotoxic effect of toxin A in the rabbit intestine appears to be due to tissue damage resulting from an inflammatory process induced by the toxin (325). Following injection of toxin A (but not toxin B), an infiltration of neutrophils into the ileum and a release of inflammatory mediators occur, causing fluid secretion, altered membrane permeability, and hemorrhagic necrosis. It has also been observed in vitro that toxin A acts as a powerful chemotactic agent for human neutrophils (256).

The cytotoxic action of toxin B appears to involve depolymerization of filamentous actin, causing a destructuring of the cell cytoskeleton and, thus, a rounding of the cell (243, 255). In light of the high potency of toxin B, the effect may be due to an indirect action, perhaps an enzymatic action on proteins involved in actin polymerization (255).

The gene for toxin A and portions of it have been cloned and expressed in *E. coli*. Muldrow et al. (212) cloned a 300-base fragment from the *C. difficile* genome that coded for a peptide serologically related to toxin A, but not active biologically. Price et al. (261) recovered a 4.7-kb DNA fragment that codes for a peptide with hemagglutinating activity corresponding to that of toxin A; this is believed to be related to the specific binding by which the toxin binds to target cells. The peptide was not cytotoxic or enterotoxic in

hamsters. Wren et al. (348) obtained a recombinant lambda phage carrying a 14.3-kb DNA fragment from *C. difficile* encoding for a 250-kDa protein that cross-reacted antigenically with antitoxin A. The lysate caused elongation (and destruction at higher concentrations) of Chinese hamster ovary cells and hemagglutination of rabbit erythrocytes. Both of these activities could be neutralized with antitoxin A and *C. sordelli* antitoxin.

ADP-ribosyltransferase (CDT). In addition to toxins A and B, a new biologically active protein with ADP-ribosyltransferase activity (CDT) has been found in a strain (CD196) of *C. difficile* (254). It was shown to modify cell actin in a manner similar to that of *C. botulinum* C_2 and *C. perfringens* iota-toxin. This protein cross-reacts antigenically with the light chains of the iota binary toxins from *C. perfringens* type E and *C. spiroforme* (253). CDT has a molecular mass of 43 kDa. Although it has no complementary binding component as have the binary toxins, it acts as a binary toxin in Vero cell cytotoxicity and mouse lethality assays when complemented by the binding peptide (iota-b) of either *C. perfringens* type E or *C. spiroforme* iota-toxins but not by the binding molecule of C_2 toxin (C_2II). CDT thus far has been found in only one strain of *C. difficile*.

CLOSTRIDIUM SORDELLII

Because of the antigenic relatedness of the toxins of *C. difficile* and *C. sordellii*, the latter species and its toxins will now be discussed.

The Organism

C. sordellii liquefies gelatin, ferments glucose, maltose, and often fructose, and produces indole, lecithinase, and urease. In peptone-yeast extract-glucose medium, it produces a major amount of acetic and lesser amounts of formic, propionic, isobutyric, butyric, isovaleric, and isocaproic acids as metabolic end products. It is distinguished from *C. bifermentans* most readily by its ability to produce urease (54, 139, 298, 345). Hall reported in 1929 (113) on the potent virulence of strains of *Bacillus sordellii* and demonstrated that pathogenicity was due to a toxin that caused severe edema and could be neutralized by a specific antiserum. For a while, *C. sordellii* was considered a subgroup of *C. bifermentans*, distinguished by its ability to produce the potent lethal toxin responsible for the severe gelatinous edema.

Diseases Caused by *C. sordellii*

The first strains of *C. sordellii* were isolated from acute edematous wound infections in humans (302). Subsequently, the organism was implicated as the cause of a fatal cattle disease in Nevada (113). MacLennan lists *C. sordellii* as one of six clostridia involved in histotoxic, gas gangrene-like infections (184). More recently, fatal *C. sordellii* infections have been noted in association with obstetrical surgical incisions (episiotomy) (195, 301). Fatality in these cases was due to clinical shock, presumably toxin induced. The described cases were characterized by sudden onset of shock with severe hypotension, generalized tissue edema, accumulation of fluids in the peritoneum and other body cavities, increased hematocrit, marked neutrophilia, absence of rash or fever, limited or no myonecrosis, and a rapid lethal course. A case of spontaneous endometritis due to *C. sordellii*, having the same rapidly fatal clinical course, has been described recently (138). A fatality due to *C. sordellii* infection in a deep laceration of a thigh has also been reported (47). Intramuscular injection of sterile fluid from cultures of *C. sordellii* isolated from the thigh wound caused edema and hemoconcentration in mice and death within 12 to 48 h, the time until death depending on dose.

Toxins

C. sordellii produces three toxins in common with the nonpathogenic *C. bifermentans*: (i) a lecithinase; (ii) an oxygen-labile hemolysin; and (iii) a fibrinolysin. The lecithinase is a phospholipase C, serologically related to the alpha-toxin of *C. perfringens* as evidenced by the positive Nagler reaction (345). It is relatively nontoxic for mice. The gene for the alpha-toxin of *C. bifermentans* has been cloned and found to be 64% homologous in coding sequence in comparison with the *C. perfringens* gene (327). It codes for a mature protein consisting of about 376 amino acids. This lecithinase from *C. bifermentans* has about 1/50 the activity of *C. perfringens* alpha-toxin in enzymatic, hemolytic, and mouse lethality assays. The calculated molecular weight, although not given for the *C. bifermentans* lecithinase, would be similar to the 43 kDa calculated for the *C. perfringens* toxin.

Major lethal toxins. A lethal factor referred to as beta-toxin (345) responsible for the pathogenicity of *C. sordellii* distinguishes it from *C. bifermentans*. Arseculeratne et al. (13) found two different activities associated with the major lethal toxin, which were separable from each other in different preparations. Both toxic factors were dermonecrotic and hemorrhagic when injected into the skin of rats and guinea pigs. The first activity (edematizing) caused massive edema and moderate areas of bright red hemorrhage when injected i.d., subcutaneously, or intramuscularly into guinea pigs, with death occurring within 24 to 36 h. The second factor (hemorrhagic) caused minimal edema but confluent areas of brownish hemorrhage in the skin and in the omentum, mesentery, and continuous viscera when injected i.v. or intraperitoneally. The hemorrhagic factor appeared to be associated with sporulating cultures. Little lethality was noted for this toxin at doses with high hemorrhagic activity.

Nakamura et al. (219) examined 55 strains of *C. sordellii* for mouse lethality and cytotoxicity. They found a close correlation between the two activities. All 34 strains negative for mouse lethal toxin lacked cytotoxin. Lethal toxin titers paralleled cytotoxicity titers. Yamakawa et al. (351) found that only 2 of 15 cytotoxigenic strains produced positive rabbit ileal loop responses (fluid accumulation). The lethal and cytotoxic effects, as well as the ileal loop response, were eliminated by preincubation of active culture fluid with either *C. sordellii* or *C. difficile* antitoxin. Two cytotoxins (cytotoxin I and cytotoxin II) were produced by one strain (3703) and were separated from each other by DEAE ion-exchange chromatography (350). The two cytotoxins could be distinguished from each other by specific antitoxins produced against each of them.

It appears that the lethal toxin classically designated as beta-toxin (345) actually consists of two toxins: lethal toxin (LT) and hemorrhagic toxin (HT). Popoff (250) purified LT from a strain of *C. sordellii* and found it to be a protein with a molecular mass of 250 kDa and an isoelectric pH of 4.55. It was antigenically related to toxin B of *C. difficile*, but not to toxin A. Martinez and Wilkins (185) purified HT and found a molecular mass of 525 kDa under nondenaturing conditions

and an isoelectric pH of 6.1. HT is cytotoxic for cultured cells and lethal for mice and causes an accumulation of hemorrhagic fluid in ligated rabbit ileal loops. It is immunologically related to toxin A of *C. difficile*. The properties and activities of HT and LT are listed and may be compared with the *C. difficile* toxins in Table 6. In summary, (i) HT is equivalent to cytotoxin I (350) and is related antigenically and in biological activity to toxin A of *C. difficile*, and (ii) LT is equivalent to cytotoxin II and is related antigenically and in biological activity to toxin B of *C. difficile*.

CLOSTRIDIUM NOVYI AND *CLOSTRIDIUM HAEMOLYTICUM*

The Organisms

C. novyi, also known previously as *C. oedematiens*, was first described by Novy in 1894 (226). It was isolated from guinea pigs who had died of "malignant oedema" after injections of casein (345). Because of the difficulties encountered in growing the organism and isolating it from wound cultures, it was not recognized as an important and frequent cause of gas gangrene in humans (184). It is now known that *C. novyi* is involved in more than one-third of such cases, and the fatality rate in would infection with this organism, especially in combination with *C. sporogenes*, is very high (298). The pathology and mortality are apparently due to the lethal alpha-toxin. The organism also is the cause of infections in domestic animals, most notably in infectious necrotic hepatitis in sheep (298). The infection follows parasitic liver infestation, usually involving the liver fluke.

C. haemolyticum is an organism so similar to *C. novyi* in physiological characteristics that it is considered by some to be a toxin variant (type D) of the latter. It is closely related to some strains of *C. novyi* (type B) by DNA relatedness criteria (215). It is the causative agent of hemoglobinuria, also known as "red water disease," a highly fatal disease of cattle (298). Diseases caused by pathogenic strains of *C. novyi* and *C. haemolyticum* appear to involve primarily the alpha-toxin of the former and the beta-toxin of the latter. In gas gangrene in humans, the alpha-toxin causes massive edema due to its effect on capillary permeability. In bacillary hemoglobinuria, the beta-toxin destroys the circulating erythrocytes, resulting in excretion of the hemoglobin in the urine; simultaneously, blood is lost into the intestine because of destruction of the capillary endothelium. The grossly different pathological presentation of these two diseases has led to maintaining two separate species for the causative organisms (54, 298).

C. novyi and *C. haemolyticum* are motile, gram-variable rods (positive in young cultures, often negative in older cultures). Cell dimensions are 0.5 to 1.6 by 1.6 to 18 μm, except for *C. novyi* type B, which are larger (1.1 to 2.5 by 3.3 to 22.5 μm) (54). They ferment glucose and liquefy gelatin. Proteolytic activity is variable. Although *C. haemolyticum* is indole positive, a few strains (notably type A) of *C. novyi* are indole negative (54, 139). All *C. novyi* strains produce acetic, propionic, and butyric acids as metabolic products.

C. novyi and *C. haemolyticum* are among the most fastidious and oxygen-sensitive bacterial pathogens known. They die rapidly on exposure to air (345). Only freshly prepared agar plates and broth media should be used to cultivate these organisms. Cooked meat-glucose has been recommended as an enrichment medium. Cultures on agar media should be incubated for at least 2 to 4 days before removal from the anaerobic system. Some strains grow too slowly to form recognizable colonies in shorter incubation times, and exposure to air may prevent further growth. On blood agar, zones of hemolysis may be seen, due to beta-, gamma-, or delta-toxins (Table 7); the beta- and gamma-toxins are "hot-cold" hemolysins; thus, the hemolytic activity is more pronounced when the plates are cooled after incubation (345). On egg yolk agar plates, opaque zones in the agar surrounding the colonies due to the lecithinase activities of beta- or gamma-toxins are apparent, and a pearly layer associated with the colony surface or its periphery is seen with strains that produce the epsilon-toxin. Spores of these organisms survive heating for 5 min at 100°C (345), so heat treatment of certain specimens and mixed cultures may be helpful for isolation of the organism.

TABLE 7. Toxins of *C. novyi* and *C. haemolyticum*[a]

Toxin	Activity	*C. novyi*			*C. haemolyticum*[b]
		Type A	Type B	Type C	
Alpha	Necrotizing, lethal	+	+	−	−
Beta	Lecithinase; necrotizing; lethal; hemolytic	−	+	−	+
Gamma	Lecithinase; necrotizing; hemolytic	+	−	−	−
Delta	Oxygen-labile hemolysin	+	−	−	−
Epsilon	Lipase (pearly layer)	+	−	−	−
Zeta	Hemolysin	−	+	−	−
Eta	Tropomyosinase	−	+	−	+
Theta	Opalescence in egg yolk	−	tr	−	+

[a] Modified from Smith and Williams (298), with permission of authors and Charles C. Thomas, Publisher.
[b] Sometimes referred to as *C. novyi* type D.

Toxin types. Classification of three types of *C. novyi* is based on production of several toxic antigenic factors. *C. novyi* types A, B, and C and *C. haemolyticum* are distinguished from each other by the toxins designated as alpha and beta (298, 345) (Table 7), based on the studies of Oakley and co-workers (228, 232, 233). Type A produces alpha only; *C. haemolyticum* produces beta only. Type B produces both. Production of six other toxins by species and toxin type is also shown in Table 7. Type C strains produce none of the known toxins and are considered nonpathogenic. Nonpathogenic strains phenotypically similar to *C. novyi* type A or to *C. botulinum* type C or D may be derived from pathogenic strains that have lost their infecting phages (54).

Genetic relatedness of *C. novyi*, *C. haemolyticum*, and *C. botulinum*. Nakamura et al. (215) examined four strains each of *C. novyi* types A and B and *C. haemolyticum* and five strains of *C. botulinum* type C. They found three groups of closely related organisms (Table 8). *C. novyi* type A is less related than either *C. novyi* type B or *C. haemolyticum* to *C. botulinum* type C. *C. novyi* type B and *C. haemolyticum* are very closely related to each other. One strain of *C. botulinum* type C (the Stockholm strain) is more closely related to the *C. novyi* type B/*C. haemolyticum* group than to the other four strains of *C. botulinum* type C.

Toxins

The lecithovitellin reaction has often been used to identify toxins of the *C. novyi* group. Rutter and Collee (273) noted that positive lecithovitellin reactions could be due to (i) lecithinase, (ii) lipase, or (iii) both lecithinase and lipase activities. They pointed out that type A strains produce gamma-toxin, a hemolytic lecithinase, and epsilon-toxin, a

TABLE 8. DNA relatedness of *C. novyi*, *C. haemolyticum*, and *C. botulinum* type C[a]

Species	No. of strains	Relation to reference strain (%)[b] from:		
		Group I	Group II	Group III
Group I				
C. novyi A	4	80–100	44–45	33–36
Group II				
C. novyi B	4	28–49	93–100	50–62
C. haemolyticum	4	26–38	84–92	44–48
C. botulinum C	1	42	85	54
Group III				
C. botulinum C	4	36–38	68–70	91–100

[a] Data condensed from Nakamura et al. (215), with permission of the senior author and the Society for General Microbiology.

[b] Values indicate relatedness between DNA from the test strain and that of the reference strain.

lipase; type B and *C. haemolyticum* strains produce beta-toxin, a hemolytic lecithinase, and also theta-toxin, which may be a lipase, though not demonstrable by a visible reaction on egg yolk agar. The beta- and gamma-lecithinases are serologically distinct. Rutter and Collee showed by thin-layer chromatography that *C. novyi* type B and *C. haemolyticum* strains break down lecithin into diglycerides, while *C. novyi* type A strains initially convert lecithin into diglycerides but subsequently break down the diglycerides from the lecithinase reaction as well as those present in the egg yolk suspension into free fatty acids and glycerol (273). Thus, *C. novyi* type A, *C. novyi* type B, and *C. haemolyticum* all possess lecithinase activity, but only type A possesses a readily demonstrable lipase. Those authors also showed a correlation between the mouse-lethal activity of the alpha-toxin of type A and B strains and cytotoxicity of the cultures.

Alpha-toxin. Phillips et al. (248) purified the alpha-toxin of *C. novyi* type B by gel filtration, adsorption onto alumina, and ultrafiltration and reported it as a protein with a molecular mass of 132 kDa. Izumi et al. (145, 146) reported a molecular mass of 260 to 280 kDa for the toxin purified by a combination of ion-exchange and gel filtration chromatography. The purified toxin had an isoelectric pH of 6.1. It had lethal, edematizing, and permeability activities but was free of the hemolytic and lecithinase activities that were present in starting culture fluid concentrate. The 50% lethal dose of the purified toxin for mice was 17 ng, and the 50% edematizing dose was 1.7 ng.

Eklund et al. (91) found that alpha-toxin production in strains of *C. novyi* types A and B was due to phages. Curing the organisms of their phages, NAI^{Tox^+} and NBI^{Tox^+}, respectively, rendered them nontoxigenic (with respect to alpha-toxin) and susceptible to reinfection by the homologous, but not the heterologous, Tox^+ phage. Reinfection resulted in their regaining toxigenicity. These phages affected only alpha-toxin production. Thus, the cured type A strain resembles nontoxigenic *C. botulinum* type C, and the cured type B strain resembles *C. haemolyticum*, since it continues to produce the beta-toxin. Schallehn and Eklund (281) infected a strain of *C. haemolyticum* with a Tox^+ phage from type A strains, rendering it capable of producing alpha-toxin; it was, thus, indistinguishable from *C. novyi* type B on the basis of the major toxins it produced.

Beta-toxin. The beta-toxin is lethal, hemolytic, and necrotizing and has lecithinase activity (345). Darakhshan and Lauerman (63) report a molecular weight of 32,000 for beta-toxin from *C. haemolyticum* purified by gel filtration chromatography. The toxin was labile at 60°C and rapidly inactivated by exposure to trypsin. The beta-toxins of *C. novyi* type B and *C. haemolyticum* appear to be serologically identical (181). Although the enzymatic activity is that of phospholipase C, it is not related serologically to *C. perfringens* alpha-toxin or to the gamma-toxin of *C. novyi* type A. *C. botulinum* type C produces a lecithinase that is comparable serologically to beta-toxin of *C. novyi* type B and *C. haemolyticum* (215). The lethality and pathogenicity of *C. haemolyticum* appear to be due to beta-toxin that is produced in large amounts by this organism, more so than by *C. novyi* type B.

Gamma-toxin. The gamma-toxin produced by *C. novyi* type A is also a phospholipase C, but is serologically distinct from beta-toxin as well as from the alpha-toxin of *C. perfringens* (180). Gamma-toxin was purified by Taguchi and Ikezawa (319) by gel filtration chromatography, and the estimated molecular mass was 30 kDa. In addition to hydrolysis of lecithin, gamma-toxin catalyzes the hydrolysis of phosphatidylinositol and phosphatidylglycerol, substrates not utilized by *C. perfringens* alpha-toxin. Beta- and gamma-toxins may be determined and differentiated by hemolytic or lipovitellin assays, using specific antitoxins (345).

Delta-toxin. The delta-toxin is an oxygen-labile hemolysin that is serologically related to similar hemolysins such as the theta-toxin of *C. perfringens* and the tetanolysin of *C. tetani* (298, 345). It is produced only by type A strains.

Zeta-toxin. Zeta-toxin is produced only by type B strains. It is not related serologically to delta-toxin or to any of the other oxygen-labile hemolysins, nor is it sensitive to oxygen (345). There is no known role for these hemolysins in the pathogenicity of the organisms that produce them.

Epsilon-toxin. Epsilon-toxin is a lipase that is responsible for the "pearly layer" associated with the bacterial colonies on egg yolk agar. This factor is seen only with type A strains and is shared in common with *C. botulinum* types C and D. The epsilon-toxin decomposes triglycerides and diglycerides in the egg yolk medium as well as diglycerides formed by hydrolysis of lecithin by gamma-toxin (273).

Eta-toxin. Eta-toxin is a proteolytic enzyme produced by *C. novyi* type B and *C. haemolyticum*. It is active on the muscle proteins, myosin and tropomyosin, and has been named "tropomyosinase" (182). Tropomyosin is hydrolyzed more completely than is myosin; 74 and 25% of the nitrogen of those respective substrates was converted to acid-soluble form. The enzyme is activated by sulfhydryl reagents such as cysteine, glutathione, and thiolactate. It is not inhibited by soybean trypsin inhibitor. It is neutralized by antisera prepared against *C. novyi* type B cultures. Its pathogenic action in muscle infection may be important, but this has not been studied (298).

CLOSTRIDIUM CHAUVOEI AND *CLOSTRIDIUM SEPTICUM*

The Organisms

C. chauvoei and *C. septicum* are similar organisms and considered by some to be members of the same species (184, 345). The major distinction is the difference in the diseases they cause. These organisms were encountered early in the history of microbiology because of their involvement in diseases of cattle and sheep (131). In the middle of the 19th

century, blackleg (Rauschbrand, Gerausch, charbon symptomatique) and malignant edema were confused with anthrax (Milzbrand, charbon). All three diseases under their various names were considered as variants of one and the same disease. Bollinger (34) reported in 1875 that numerous short bacteria observed microscopically in tissues from cattle dying from Gerausch had no similarity whatsoever with the filamentous anthrax bacillus. Arloing, Cornevin, and Thomas described the blackleg organism in some detail in 1880 (157). The name *Clostridium chauvoei* was given in honor of the French veterinarian Auguste Chauveau (345).

C. chauvoei is primarily involved in blackleg, or Rauschbrand, a gas gangrenous infection in cattle and sheep. Blackleg appears to be a nontraumatic endogenous infection in cattle, since infection of muscle tissue occurs in the absence of a wound or a break in the skin (298). The possibility of an insect vector has been considered (131). In sheep, blackleg often is due to *C. chauvoei* infecting a wound caused by shearing, castrating, or docking. The disease progresses rapidly, with the animal dying in 12 to 36 h after the appearance of the first symptoms. The affected muscles are dark in color, and edema is restricted to the local area.

In 1877, Pasteur related his observations on anthrax that, after death, the anthrax bacillus in the blood of an animal disappears because of lack of oxygen and the blood becomes toxic because of the growth of anaerobic putrefactive organisms originating in the intestines (245). Inoculation of blood taken belatedly from such animals into guinea pigs caused death other than by anthrax. An acute inflammation of the abdominal muscles and of the paws and pockets of gas in the axillar regions were evident. Motile anaerobic organisms were found in muscle tissues throughout the body and in the massive fluid accumulations in the body cavities. Greatly elongated bacilli were found on the surface of the visceral organs. Upon contact with air, the organisms lost their motility; they did not die, but appeared to transform themselves into the spore state. The organism was named *Vibrion septique*. After a history of confusion due to studies on mixed cultures and misidentified cultures (131, 345), the organism became known as *Clostridium septicum*. *C. septicum* causes a disease in sheep known as braxy (41). The organism invades vulnerable mucosa and submucosa of the abomasum, multiplies, and produces a fatal bacteremia and toxemia. Death follows within a few hours of first observation of symptoms. *C. septicum* has also been found as a cause of wound infections in cattle, usually recognized and distinguished from blackleg by the designation malignant edema (298). This infectious condition is highly fatal within 1 or 2 days of the first symptoms and is characterized by rapidly progressing subcutaneous swelling. Fluid accumulation in the body cavities is often noted at autopsy. Infected muscles are deep red. The generalized edema and different appearance of the affected muscles clearly distinguished *C. septicum* infections from those of *C. chauvoei*.

C. septicum was found as a major cause of gas gangrene due to infection of war wounds (184). More recently, it has been found in infections associated with malignancy, and the source of the organism is believed to be the patient's own intestinal tract (8, 247). Evidence tends to implicate *C. septicum* as the causative agent of neutropenic enterocolitis (38). The pronounced edema and intestinal wall thickening, with necrosis and hemorrhage at the site of infection in the cecum or ileum, are consistent with toxins produced by this organism. *C. septicum* exclusively has been found in the bowel tissue in some thoroughly investigated cases (156).

C. chauvoei and *C. septicum* are difficult to distinguish from each other on the basis of their physiological and toxigenic characteristics. Both are rather oxygen tolerant and easy to culture, although they may be difficult to isolate because of their vigorous motility and tendency to swarm on the surface of agar media (345). The organisms are gram-positive rods (often gram negative in older cultures). The cell dimensions listed for *C. chauvoei* are 0.5 to 1.7 by 1.6 to 9.7 μm and are slightly greater for *C. septicum*, 0.6 to 1.9 by 1.9 to 35 μm, indicating that more pleomorphism of the latter has been observed. Both organisms ferment glucose, fructose, lactose, maltose, and mannose. They liquefy gelatin but do not digest meat or milk proteins. Both produce acetic and butyric acids as metabolic end products. A differential characteristic that is widely accepted is sucrose fermentation, which is positive for *C. chauvoei* and negative for *C. septicum* (54, 139), but Al-Khatib (6) has reported that this distinction is unreliable. It has also been reported that long chains of bacilli or long filaments in the serous cavities and on the liver surface in infected animals are formed by *C. septicum* but not by *C. chauvoei* (131, 345). This means of differentiation is also disputed by Al-Khatib (6).

It appears that the only reliable means of differentiation are serological, pathological, or toxicological (131). Moussa studied 37 strains of *C. septicum* and 38 strains of *C. chauvoei* serologically and found eight different serological groups (210). Five groups contained only *C. septicum* and two groups contained only *C. chauvoei*. The one mixed group contained only one strain of *C. septicum* and two of *C. chauvoei*. With the exception of the mixed group, the two species can be distinguished on the basis of their somatic antigens. *C. septicum* strains possess O-antigen 1 or 2, while *C. chauvoei* strains possess O-antigen 3. Within each O-antigen group, strains may be differentiated on the basis of their H (flagellar) antigens. A common spore antigen was found for all strains regardless of species designation (210). The O-antigen differences provide the basis for distinguishing the two organisms by using specific fluorescent-antibody reagents (19). Vaccines for protection of animals against infection by *C. chauvoei* (183) and *C. septicum* (60) appear to be effective, but protection by *C. septicum* vaccines is somewhat strain specific and may be related to the antigenic diversity of those organisms, as indicated by Moussa's studies (210).

Toxins

C. chauvoei and *C. septicum* produce four major toxins that correspond to each other in biological activity (298, 345).

Alpha-toxin. Information on the alpha-toxins of these two organisms is somewhat vague and confusing. Bernheimer (25) and others established that the alpha-toxin of *C. septicum* is responsible for the lethal and necrotizing activities of the culture filtrates and also for at least some of the hemolytic activity. Verpoorte et al. (339) reported that *C. chauvoei* alpha-toxin has a molecular mass of 27 kDa, but is formed as part of a larger 53.5-kDa complex, which is referred to as the "soluble immunizing component" (298). *C. chauvoei* does not produce titers of alpha-toxin as high as *C. septicum*, and with cultures of the former it is sometimes difficult to detect. Al-Khatib (5, 6) found that antiserum to *C. septicum* neutralized the lethal activity of both organisms, but antiserum to *C. chauvoei* neutralized the lethality of the homologous organism only. He postulated that *C. septicum* produces two alpha-toxins, alpha-1 and alpha-2, while *C. chauvoei* produces only alpha-1 (6). Moussa (209) also found

that *C. chauvoei* antisera failed to neutralize *C. septicum* alpha-toxin.

Beta-toxin. The beta-toxin from these organisms is a DNase. Princewill and Oakley (262) found that the beta-toxins of both species were similar with regard to heat stability, activation or inhibition by metal ions, inhibition by chelating agents, and precipitability with ammonium sulfate. They are quite heat stable, with only about 10% loss of activity after heating at 56°C for 60 min and 50% loss after heating at 100°C for 80 min. Enzyme activity is enhanced by added barium or magnesium ions and inhibited by copper, iron, mercury, zinc, or silver ions. The activity was not affected by concentrations of calcium, cobalt, magnesium, potassium, or sodium ions between 10^{-1} and 10^{-6} M. Neutralization tests showed that the beta-toxins from the two organisms are not identical. Although some *C. septicum* antitoxins neutralized *C. chauvoei* beta-toxin, some of the antisera from *C. septicum* and all antisera from *C. chauvoei* neutralized only the homologous beta-toxin (262). Moussa (209) found cross-neutralization with all sera from both species. *C. septicum* antisera neutralized the beta-toxin of both species to a similar degree, while *C. chauvoei* antisera were more potent in neutralizing the homologous beta-toxin. Swiatek et al. (318) report that all 62 strains of *C. septicum* they examined produced beta-toxin and that the levels of DNase activity of those strains were much higher than those produced by any of the other clostridia they tested. *C. chauvoei* was not included in their study. They estimated a molecular mass of 45 kDa for the enzyme. Beta-toxin can be assayed by the acid-Congo red-alcohol test, using DNA as the substrate (230).

Gamma-toxin. The gamma-toxin from these organisms is a hyaluronidase, an enzyme that hydrolyzes glycosidic bonds between *N*-acetylglucosamine and glucuronic acid residues of hyaluronic acid. This enzyme is readily inactivated by heat treatment. Princewill and Oakley (262) found that the gamma-toxin of *C. septicum* was much more sensitive to heat than was that of *C. chauvoei*. Enzyme activity was affected by metal ions in the same way as the beta-toxin, except that it was stimulated by calcium in addition to barium and magnesium. Cross-neutralization of the toxins by antisera of both organisms has been shown (6, 209), although Princewill and Oakley (262) failed to find gamma-toxin neutralization with any of their *C. chauvoei* sera. Gadalla and Collee (95) found that gamma-toxin (as well as neuraminidase) was produced in large amounts in embryonated hen eggs and muscle tissues of animals infected with *C. septicum*. It was also found in the edematous fluid of infected animals. The acid-Congo red-alcohol test can be used to assay gamma-toxin by using hyaluronic acid as the substrate instead of DNA as used to assay beta-toxin (230).

Delta-toxin. The delta-toxin produced by these organisms is an oxygen-labile hemolysin similar to counterparts produced by other clostridia (e.g., the theta-toxin of *C. perfringens* and tetanolysin of *C. tetani*). It is neutralized by antistreptolysin-O serum (209). The delta-toxins of *C. chauvoei* and *C. septicum* are cross-neutralized by their opposite antisera, but the neutralizing potency of the homologous antisera is clearly stronger. The hemolytic potency of the toxins is inactivated by oxidizing agents such as hydrogen peroxide and iodine; the inactivation can be reversed by treatment with reducing agents such as sodium thioglycolate and hydrogen sulfide. Hemolysis produced by the delta-toxin occurs more rapidly than that produced by the alpha-toxin. Neutralization of the delta-hemolysin with antistreptolysin O allows the demonstration of the alpha-hemolysin alone (209).

Neuraminidase. In addition to the above four toxins, *C. septicum* has been shown to produce considerable amounts of neuraminidase in culture as well as in infected embryonated eggs and animal tissues (95). It has been suggested that the neuraminidase which alters glycoproteins on cell membranes and mucoid secretions may render target sites vulnerable to attack by other toxins produced by the organism.

CLOSTRIDIUM HISTOLYTICUM

The Organism

C. histolyticum is of interest because of its isolation from gangrenous and nongangrenous war wounds, its production of a lethal toxin that cross-reacts serologically with the alpha-toxin of *C. septicum*, and its production of a mixture of collagenases and other proteolytic enzymes that are unique in their efficiency in converting tissue proteins to amino acids and peptides. The organism was first described by Weinberg and Seguin in 1916 (341). Its isolation from wounds and implication as a cause of gas gangrene are rather infrequent (184, 298, 345). However, it may play an important role as a component of a virulent mixture of organisms, e.g., in combination with *C. perfringens* in gas gangrene infections (341). Its infrequent isolation may be due to certain difficulties in culturing the organism. Although it is not a strict anaerobe (it may grow on agar media in the presence of air) and its nutritional requirements are not more stringent than those of many other clostridia, its growth may be inhibited by the presence of sugar in the medium and, contrary to some reports (260, 354), the heat resistance of spores may be rather low (223).

C. histolyticum is a gram-positive rod, 0.5 to 0.9 by 1.3 to 9.3 μm, appearing singly, in pairs, or as short chains. It is asaccharolytic and strongly proteolytic, but not unique, as *C. subteminale*, *C. hastiforme*, and *C. argentinense* are also asaccharolytic and proteolytic (54, 313). It is unusual among proteolytic clostridia in not producing isoacids (isobutyric and isocaproic acids) as metabolic end products. Only acetic acid is formed (139). It digests casein, gelatin, hemoglobin, albumin, collagen (298), and elastin, the latter by means of its delta-toxin (345). Little or no gas is detected in cultures. It is negative for the lecithinase and lipase reactions. Thus, with the exception of the protein reactions, all commonly used culture tests are negative.

Toxins

C. histolyticum produces five toxins, designated by the first five letters of the Greek alphabet, in the order of their recognition. It is pathogenic for laboratory animals (345). Strains that produce appreciable amounts of alpha (lethal)-toxin cause death of a laboratory animal within 24 h of intramuscular injection of the organism. In the absence of alpha-toxin, no toxemia ensues, but a progressive gangrenous infection may take place, with tissue destruction due largely to actions of the organism's collagenolytic and proteolytic toxins. The skin over the area of infected muscle is completely destroyed and the underlying tissues are grossly digested, as are also the soft parts of the bones (2). Ultimately, the digestive process may terminate with autoamputation of the infected limb, but in some cases infection may spread into the trunk of the body, damaging vital organs and resulting in death (341, 345).

Alpha-toxin. Lethal toxicity of culture filtrates of *C. histolyticum* was reported by Weinberg and Seguin (341). This lethal factor was designated alpha-toxin and noted to be necrotizing but not hemolytic (229). It has been noted that the alpha-toxin of *C. histolyticum* can be neutralized by antisera produced against toxic filtrates of *C. septicum* cultures; similarly, the lethality of *C. septicum* cultures can be neutralized by *C. histolyticum* antisera (305). Cross-neutralizations can be demonstrated in both mouse lethality and i.d. necrosis reactions.

Nishida and Imaizumi (223) found that only 6 of 21 strains of *C. histolyticum* isolated from soil samples in Japan produced alpha-toxin in culture. The pharmacological and molecular properties of this toxin have not been studied in depth, probably because it is rather unstable. It is readily inactivated by proteolytic enzymes (298). Bowen (45) reported 85 50% lethal doses per mg of toxin per kg of body weight for mice for alpha-toxin partially purified by ethanol precipitation.

Beta-toxin. Beta-toxin has been identified as a collagenase (EC 3.4.24.3) or more recently as a group of seven collagenases (35, 335). Collagenase is a zinc metalloprotease that cleaves native triple helix collagen as well as gelatin into small fragments (74). The substrate, collagen, is the most abundant protein in the animal body, constituting one-fourth to one-third of the total protein forming the insoluble fiber of connective tissue (343). Yoshida and Noda (353) first separated *C. histolyticum* collagenase into two fractions designated as I and II. While both degraded collagen at similar rates, collagenase II split more peptide bonds in the collagen molecule and was more efficient in degrading lower-molecular-weight polypeptides. Van Wart et al. (35, 36, 335) have identified seven distinguishable molecular forms of *C. histolyticum* collagenase and designated each with a Greek letter; unfortunately, five of these can be confused with the five toxins of this organism designated by the same Greek letters. Each collagenase is distinguished by its molecular mass, determined as 68, 115, 79, 100, 110, 125, and 130 kDa for collagenases alpha, beta, gamma, delta, epsilon, zeta, and eta, respectively. Two subspecies of the alpha- and gamma-collagenases are noted on the basis of different isoelectric points. Class I collagenase activity is found with the alpha, beta, gamma, and eta molecular species, and class II is found with the delta, epsilon, and zeta species. The class I collagenases have extensive amino acid sequence homology with each other, and similar homology is found among the class II enzymes, but the two classes have substantially different sequences (37). Evidence shows intragenic duplication in the beta molecule, which may account for its higher molecular weight. This duplication may be true also for the other higher-molecular-weight collagenases. The collagenases are all related serologically, but some distinctions are evident by immunodiffusion analysis in agar gels. The two classes of collagenases have been shown to have similar but complementary substrate amino acid sequence specificities by which they synergistically digest collagen (205, 335). Beta-toxin no doubt plays a major role in the pathology of *C. histolyticum* infections in view of its ability to destroy collagen fibers. The toxin induces hemorrhage when placed on the surface of the lungs of animals and causes hemorrhage and edema when injected into rat paws (336). It causes a lethal intrapulmonary hemorrhage when injected i.v. (298).

Gamma-toxin. Gamma-toxin is a thiol-activated proteinase that digests hide powder, azocoll, gelatin, and casein, but is inactive against collagen (345). Its molecular weight is about 50,000 (298).

Delta-toxin. The delta-toxin is also a proteolytic enzyme, but more specifically, it is an elastase (345). It is reversibly inactivated by reducing agents. Takahashi et al. (320) partially purified delta-toxin and found that it passed through a 50-kDA ultramembrane filter but was retained by a 10-kDa membrane. The active fraction obtained by gel filtration and ion-exchange chromatography had high specific caseinolytic as well as elastolytic activity. It was not active on collagen. The elastolytic and caseinolytic activities were not affected by chelating agents.

Epsilon-toxin. The epsilon-toxin is an oxygen-labile hemolysin similar serologically to those produced by other clostridia such as *C. tetani*, *C. septicum*, and *C. novyi* (26, 298, 345).

CLOSTRIDIUM SPIROFORME

The Organism

Since 1964, there have been several reports of helically coiled, gram-positive, anaerobic organisms isolated from animal feces and cecum contents (32, 94). Because spore formation was noted in some of these strains, they were recognized as clostridia (160). Kaneuchi et al. (154) compared 33 strains of coiled sporeforming organisms recovered from humans and other animals. All of the organisms were nonproteolytic and nongelatinolytic, fermented glucose, and produced terminal to subterminal round spores. Two main groups were established on the basis of physiological characteristics and DNA relatedness; 21 strains were designated as *C. cocleatum* and 8 strains were designated as *C. spiroforme*. Each of these two species have 46 to 60% relatedness with reference strains of the opposite species and 74 to 100% relatedness in comparison to strains within each species. The phenotypic differences noted by Kaneuchi et al. (154) were fermentation of galactose by *C. cocleatum* and a more pronounced coiling by *C. spiroforme*. Both species were related to *C. ramosum*: 35 to 52% for *C. cocleatum*, and 32 to 53% for *C. spiroforme*. *C. ramosum* has a straight rod morphology and has alpha-methylmannosidase activities that are not found in the two coiled organisms.

After *C. spiroforme* cultures are heated at 80°C for 10 min, uncoiled straight cells are found that do not revert to the coiled structure upon subculture (154). No alterations of any other features of the organism are observed. The helical structure of the native organism consists of numerous single semicircular cells joined end-to-end (42). The individual cells are nonmotile and gram positive, with dimensions of 0.3 to 0.5 by 2.0 to 10 μm (54).

Much clinical interest in *C. spiroforme* arose when an organism with similar morphological and physiological characteristics which produced a toxin neutralizable with type E antitoxin of *C. perfringens* was consistently isolated from feces of scouring rabbits (51). Borriello and Carman (40) reported complete agreement between the characteristics of toxigenic isolates from rabbits and the type strain of *C. spiroforme*, but Moore et al. (206) question the validity of the identity of the toxigenic strains. Since most of the existent literature considers the toxigenic organisms to be *C. spiroforme*, they are presented as such in this review, even though they may be classified otherwise in the future. Iota-toxin had been found previously in rabbit enterotoxemia, but investigators had attempted to recover iota-toxigenic *C. perfringens* (18, 246). The toxigenic organism

identified as *C. spiroforme* was subsequently implicated as the cause of both spontaneous and antibiotic-induced diarrhea and colitis in rabbits (40, 52). Holmes et al. (140) found that isolation of the organism from rabbit intestinal contents can be facilitated by high-speed centrifugation (20,000 × *g*) of cecal contents and culturing the material at the supernatant-pellet interface. The helical shape of the organism perhaps is responsible for its slower sedimentation than that of other microorganisms in the samples and, thus, its accumulation on the top of the pellet.

Iota-Toxin

The iota-toxin of *C. spiroforme* is a binary toxin with components iota-a and iota-b that correspond serologically and electrophoretically to the components of *C. perfringens* iota-toxin (309). The electrophoretic mobilities of the latter are slightly faster. While the *C. spiroforme* antiserum formed precipitates with both components of the toxin from either species, *C. perfringens* antiserum formed visible precipitates only with the b component of the *C. spiroforme* toxin on immunoelectrophoretic plates.

Popoff and Boquet (251) purified the a component of *C. spiroforme* iota-toxin by immunoaffinity and reported that it consists of a heterogeneous population of molecules with molecular masses ranging from 43 to 47 kDa. This is similar to the 47.5-kDa size of the iota-a component of *C. perfringens*. This component has ADP-ribosyltransferase activity towards actin. It was inactive in both cytotoxic and mouse lethality assays by itself but it was potentiated by the component iota-b from *C. spiroforme* or *C. perfringens*. Iota-b from *C. spiroforme* potentiated iota-a from *C. perfringens* and also on ADP-ribosyltransferase (CDT) from *C. difficile* in those same assays. The components of the binary C_2 toxin of *C. botulinum* did not synergize with any of the binary components of the other three species.

MISCELLANEOUS SPECIES

Clostridium butyricum

Neuraminidase is the only toxin usually noted for *C. butyricum* (252). An association of this organism with neonatal necrotizing enterocolitis has been observed in numerous studies (141, 158, 167). *C. butyricum* is also often found in stools of normal infants. Cytotoxicity, noted with necrotizing enterocolitis-associated strains, has been considered as the possible virulence factor for the pathology of the illness. Popoff et al. (253) found that all 37 strains of *C. butyricum* isolated from sick as well as healthy newborns produced a cytotoxic substance in culture. They identified the factor as butyric acid, a major end product of *C. butyricum* metabolism. They concluded that short-chain carboxylic acids, mainly acetic and butyric, in high concentrations might play a role in the disease in newborns. Aureli et al. (14) and McCroskey et al. (192) documented two cases of type E infant botulism due to neurotoxigenic *C. butyricum*. The identity of the organism was confirmed by DNA relatedness studies (313), and the characteristics of the purified toxin from this organism were totally consistent with those of botulinal neurotoxin from *C. botulinum* type E (101).

Clostridium baratii

C. baratii has attracted only limited attention because of its lack of overt pathogenesis and its failure to produce any lethal toxins. Its reactions in biochemical tests are very similar to those of *C. perfringens*, thus, the former synonym, *C. paraperfringens*. This organism produces a lecithinase reaction on egg yolk agar that is weakly neutralized by *C. perfringens* antiserum (218). The major distinguishing phenotypic characteristic is its failure to liquefy gelatin (54). It has been isolated from normal infant stools (303), as well as from adult human and rat feces, war wounds, other clinical specimens, and from soil and sediment samples (54). The most striking finding with this organism was the implication of a neurotoxigenic strain as the cause of type F infant botulism (115, 137). DNA relatedness studies confirmed the relationship of the neurotoxigenic strain to the type strain of *C. baratii* (312). Subsequent isolation of a neurotoxigenic strain of *C. baratii* from an adult indicates that more such strains exist (L. M. McCroskey and C. L. Hatheway, unpublished laboratory findings). If they are able to colonize the intestinal tract, they have the ability to cause botulism.

Other species of *Clostridium* produce factors such as lecithinase, lipase, DNase, and hemolysins that are treated under the term toxin, but since the strains are generally nonpathogenic and do not produce lethal toxins, they will not be discussed here.

SUMMARY

The toxins discussed here are antigenic factors with demonstrable biological activities, produced by clostridia that are pathogenic to humans or other animals. Many but not all of the toxins have known roles in the pathology of the diseases caused by the organisms. Toxins with the same or similar activities and molecular properties are often common to different clostridial species or groups. Most notable among these are the neurotoxins, "lethal toxins," lecithinases, oxygen-labile hemolysins, and the binary ADP-ribosyltransferases. These biologically active factors and the organisms that produce them are listed in Table 9. Other factors with activities less fascinating at the moment, such as the lipases, proteinases, neuraminidases, hyaluronidases, and DNases, no doubt serve the organisms in their metabolic and pathogenic processes and provide differential features for taxonomic purposes.

Neurotoxins

The tetanus and botulinal neurotoxins are very similar in molecular structure. They are synthesized as single peptides with one or more intramolecular disulfide bridges. Cleavage of a peptide bond forms the fully active toxic bichain, consisting of a heavy chain (100 kDa) and a light chain (50 kDa) joined by a disulfide bridge. Compared with seven serological types of botulinal toxin, only one serological type of tetanus toxin has been identified. The mechanisms of action of tetanus and botulinal toxins have similarities and differences. Both bind to a receptor on nerve cell membranes. Different receptors seem to serve different types of botulinal toxin. Type B may have a receptor affinity more similar to tetanus toxin than to type A toxin. The binding site for all neurotoxins is on the heavy chain of the toxin. The paralytic step requires the internalization into the nerve cell of a fragment which includes the light chain or a portion of it. All cause muscular dysfunction by blocking neurotransmitter release. Much of the tetanus toxin reaches the central nervous system by retrograde transport up the nerve axon, while the botulinal toxins exert their activity mainly at the nerve terminus at the muscle fiber.

TABLE 9. Some clostridial toxins common to different species

Species	Neurotoxin	Lethal, edematizing, necrotizing	Phospholipase C (lecithinase)	O_2-labile hemolysin	ADP-ribosylating factor
C. tetani	T			Tetanolysin	
C. botulinum					
I	A, B, F				
II	B, E, F				
III	C, D		±	+	C_2 (binary), C_3
C. argentinense	G				
C. perfringens					
A			Alpha	Theta	
B		Beta, epsilon	Alpha	Theta	
C		Beta	Alpha	Theta	
D		Epsilon	Alpha	Theta	
E			Alpha	Theta	Iota (binary)
C. novyi					
A		Alpha	Gamma	Delta	
B		Alpha	Beta		
C					
C. haemolyticum			Beta		
C. difficile		Toxin A, toxin B			CDT[a]
C. sordellii		Beta (LT, HT)	Gamma	+	
C. septicum		Alpha		Delta	
C. chauvoei		Alpha		Delta	
C. histolyticum		Alpha		Epsilon	
C. spiroforme					Iota (binary)
C. butyricum	E				
C. baratii	F		+		

[a] CDT of *C. difficile* will synergize with b components of the iota-toxins of *C. perfringens* and *C. spiroforme* (254).

The structural differences in the molecules responsible for the difference in action of tetanus and botulinal toxins have not been determined. Remarkable amino acid homology is found in the known sequences of the light and heavy chains of type B botulinal toxin and tetanus toxin: 31 of 44 (70%) and 13 of 26 (50%) for the light and heavy chains, respectively (64). Eisel et al. (82) noted that amino acid similarities among type A, B, and E botulinal toxins and the tetanus toxin suggest that they stem from a common ancestral gene.

The genes for tetanus (93) and type G botulinal (89) toxins appear to be carried in plasmids, while those for type C and D botulinal toxins have been shown to be contained in phage genomes (90). The location of the genes for types A, B, E, and F has not been established. Experimental interspecies transfer of genes for type C and D toxin has been demonstrated (88, 90). The discoveries of neurotoxigenic *C. butyricum* (192) and *C. baratii* (115) give rise to speculation on interspecies transfer in nature of genes for type E and F toxins. The complete sequences of the tetanus toxin gene and of the toxin molecule have been determined by cloning of overlapping gene segments (82). Determining the sequences for the botulinal toxins no doubt awaits application of the same techniques. Comparative studies of the complete structures may provide the means of understanding their functional similarities and differences.

Lethal toxins

Initially, most of the toxins of the pathogenic clostridia were evident because of their lethality for animals. They were then identified by using toxin neutralization tests with specific antisera. Subsequently, many of the lethal toxins were purified and their physical and biochemical properties were determined. The alpha-toxins of *C. novyi*, *C. chauvoei*, *C. septicum*, and *C. histolyticum* have thus far remained poorly defined. Perhaps some of these toxins are similar to lethal toxins produced by *C. perfringens* (beta and epsilon), *C. difficile* (toxins A and B), and *C. sordellii* (HT and LT). Before the role of *C. difficile* in antibiotic-associated pseudomembranous colitis was recognized, its toxins were as poorly defined as the alpha-toxins mentioned. The *C. sordellii* beta-toxin complex has been studied and more clearly defined because of the cross-neutralization of the *C. difficile* toxins by *C. sordellii* antitoxin. Evidence suggests that *C. septicum* alpha-toxin has more than one component. Al-Khatib (6) suggests that *C. septicum* produces alpha-1 and alpha-2, and that *C. chauvoei* produces only alpha-1, on the basis of differential cross-neutralization results. At present, these lethal toxins offer interesting potential for research on clostridial toxins.

Lecithinases

The prototype of the clostridial lecithinases is the alpha-toxin of *C. perfringens*, which provides the basis of the Nagler reaction (214, 218). Enzymatically, *C. perfringens* alpha-toxin behaves as a phospholipase C. Lecithinases produced by *C. sordellii*, *C. bifermentans*, and *C. baratii* are inhibited to some extent by the *C. perfringens* antiserum, and those produced by *C. novyi* types A and B and *C. haemolyticum* are neutralized by their homologous antisera but not by *C. perfringens* antiserum. The lecithinases of *C. novyi* type B and *C. haemolyticum* (beta-toxin) can be distinguished serologically from that of *C. novyi* type A (gamma-toxin) (345). Although *C. sordellii*, *C. bifermentans*, and *C. baratii* are Nagler positive, the neutralizations are less sharp, probably due to (i) the amount of lecithinase produced by the organism, (ii) the specific activity of the enzyme, and (iii) the avidity of the antibodies for the protein molecule. Tso and Seibel (327) found 64% homology in the coding sequence of the genes coding for the alpha-toxins of *C. perfringens* and *C. bifermentans* and a 50-fold-lower activity of the latter toxin. Researchers will probably want to clone the lecithinase genes from other clostridia.

Oxygen-Labile Hemolysins

Oxygen-labile hemolysins produced by clostridia are designated tetanolysin (*C. tetani*), theta-toxin (*C. perfringens*), delta-toxin (*C. novyi* type A, *C. septicum*, and *C. chauvoei*), and epsilon-toxin (*C. histolyticum*). They lyse a variety of cells in addition to erythrocytes. These cytolysins are serologically related to each other and to streptolysin O, pneumolysin, cereolysin, thuringiolysin, aveolysin, laterosporolysin, and listeriolysin, produced by bacteria in other genera (26). They are inactivated by mild oxidizing conditions, reactivated by thiol compounds, and inhibited by a small amount of cholesterol. Bernheimer (26) also includes *C. bifermentans* (and *C. sordellii*) and *C. botulinum* types C and D among the organisms that produce these hemolysins. The gene for *C. perfringens* theta-toxin has been cloned, and the amino acid sequence of the protein is now known (330, 331). Streptolysin O and pneumolysin possess considerable homology, with a common identical 12-amino-acid segment. Oxygen-labile hemolysins have been shown to have lethal and cardiotoxic effects in vivo and to cause intravascular lysis (26, 117). The role they play in diseases caused by the organisms that produce them is difficult to assess. With such a widespread occurrence of these cytolysins in bacteria, they must serve some advantageous function.

Binary Toxins and ADP-Ribosylating Factors

Although the iota-toxin of *C. perfringens* (44) and the C_2 toxin of *C. botulinum* types C and D (147, 148) have been shown for many years, their binary nature and ADP-ribosylating activity have been recognized only very recently. In 1980, Iwasaki et al. (144) showed that C_2 toxin consisted of two separate proteins, C_2I and C_2II, both of which were necessary for lethal and vascular permeability (239), and enterotoxic activities (236). It was later found that component I was an enzyme that possessed ADP-ribosylating activity (291). This enzyme transfers the ADP component of NAD to nonmuscle actin (45 kDa) but not skeletal muscle actin, attaching it by an *N*-glycosyl bond to Arg-177 in the actin molecule (332). ADP-ribosylating activity of clostridial toxins is of great interest because it may explain the molecular basis for the effect of the toxin on host cells. This effect has been true for diphtheria and cholera toxins as well as others (10). Speculation for such a mechanism for neurotoxins has been discussed (C. Montecucco, B. Bizzini, and L. L. Simpson, letters, Toxicon **25**:1255–1263, 1987).

Several research groups reported ADP-ribosylating activity for *C. botulinum* type C and D neurotoxins (15, 186, 234, 235). Subsequent evidence showed that the ADP-ribosylating activity in the neurotoxin preparations was due to another factor, C_3, which was present as a contaminant (266). C_3 is a novel ADP-ribosyltransferase, distinct from C_2 toxin (4), which ADP-ribosylates 21- to 24-kDa guanine nucleotide binding proteins from various eucaryotic cells (266). C_3 is not a binary toxin and thus cannot gain entry to a cell by synergizing with a membrane-binding component. Since it has no toxic activity in itself, some researchers refer to it as an "exoenzyme" instead of a toxin (272). The production of C_3 is associated with the production of the type C or D neurotoxin. The gene may be included in the genome of the phage carrying the neurotoxin gene, but evidence has failed to show that C_3 is derived from the neurotoxins (272).

The iota-toxin of *C. perfringens* was also shown to be a binary toxin with subunits similar to the C_2 toxin (308), and the light chain in this case also possesses ADP-ribosylating activity (294). The substrates for this enzyme are both muscle and nonmuscle actin, and the site for both substrate molecules is Arg-177 (332). The binary nature of the *C. spiroforme* iota-toxin was also confirmed (309), and serological cross-reactions between the corresponding components of the iota-toxins of *C. perfringens* and *C. spiroforme* were observed.

An ADP-ribosyltransferase (CDT) has been found in one strain of *C. difficile* (254). The substrate for this enzyme is cell actin. No complementary binding component was produced by the organism; thus, CDT is similar to C_3 in this respect, but similar to C_2I in substrate specificity. Popoff and Boquet (251) have shown that the binding components of the iota-toxins of *C. perfringens* and *C. spiroforme* would complement the ADP-ribosylating components of either species, forming potent toxic combinations in both mouse lethality and Vero cell toxicity assays. The binding component of either iota-toxin will also complement the CDT enzyme in both assays. The binding component of C_2 toxin (C_2II) was not able to complement the ADP-ribosylating components from *C. perfringens*, *C. spiroforme*, or *C. difficile*.

Final Comments

As can be seen in this review, the toxins produced by clostridia encompass a broad range of bacterial proteins. Many of them play a prominent role in producing the pathological effects caused by the organism. For many of the clostridia, the manifestations of the toxins overshadow all of the other physiological and genetic properties of the organisms. This is evident in the taxonomic problems or enigmas we are encumbered with today. The toxins produced by the various species of *Clostridium* provide scientists with interesting and useful materials for studying disease mechanisms, specific immunities, neurological function, cellular processes, molecular structures, and the genes that code for the proteins. The vast amount of literature on the subject attests to the great interest these toxins have attracted. I reiterate in this conclusion the suggestion that a less confusing, more descriptive systematic nomenclature system for clostridial toxins and other biologically active clostridial proteins be devised by an appropriate panel of experts.

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